CHARACTERIZATION OF THE ENTRY MECHANISMS OF JUNÍN ARENAVIRUS

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Junín Arenavirus causes Viral Hemorrhagic Fever with a 30% fatality rate in those infected. Yet the exact pathogenesis of disease is still unknown. To date, the only known human receptor is transferrin (TfR1). TfR1 is located basolaterally in the lung epithelium and unavailable to inhaled virus particles. One proposed mechanism for viral infection and pathogenesis is that antigen presenting cells may interact with the virus and become infected, Here we show that immature dendritic cells (DCs) can interact with the Junín pseudovirus virus (JUNVpp) but do not become productively infected. Instead they harbor the virus like a Trojan Horse in a process called *trans*-infection. Dendritic cells have both the TfR1 receptor and an antigen processing receptor, DC-SIGN. The virus associates with both receptors and early endosomal compartment but was not seen associating with late endosomal compartments where fusion of the viral envelope with the host cell membrane would normally occur. *In-vivo*, a trans-infected DC would travel to a lymph node. We utilized a primary human sinusoidal endothelial cell (HHSEC) which possesses some of the same receptors as lymph nodes, mainly DC-SIGN, L-SIGN and LSECtin. JUNVpp can utilize LSECtin as a receptor. DC-SIGN and L-SIGN also seem to function as entry receptors in the context of HHSEC cells though to a lesser extent than TfR1 and LSECtin.

Since viruses can interact with receptors through glycosylation sites on the glycoprotein we analyzed each glycosylation site on the GP-1 portion of the glycoprotein by mutating the critical asparagine to a glutamine. The N-linked sites are not involved in binding and entry with the TfR1 receptor and deficits in entry can be attributed to structural changes or inability to use a C-type lectin as a receptor. All sites were important for maintaining structure as determined by reduced ability of the mutants to associate with the TfR1 receptor. Some mutants had defects in cleavage and were not incorporated into the virion. Our results suggest that N-linked glycosylation sites are critical for virus structure and eventual binding with the C-type lectins LSECtin, DC-SIGN and L-SIGN.

BIOGRAPHICAL SKETCH

Michele Annette Bialecki was born in Williamsport, Pennsylvania in 1966. She lived in North and South Carolina until eventually moving back to Pennsylvania. Michele always had a knack for science and in 1982, as a sophomore, was named the Female Champion of a Science Olympiad held at Mansfield University. She was the Valedictorian of her graduating class from Liberty High School in 1984.

In 1998, Michele decided to attend college. Once again because of her knack for and interest in science, she was hired as a Laboratory Technician. She also tutored most science classes. During her time at Lock Haven University, she received an NSF sponsored fellowship, the Biogeochemical Research Initiative for Education. She labeled antibodies with ferromagnetite particles which were used to label bacteria that were injected into core samples. The bacteria were then visualized with X-ray computed micro-tomography.

Michele graduated summa cum laude from Lock Haven University in 2003 with a Bachelor of Science in Cellular and Organismal Biology with a minor in Chemistry. She also obtained a Bachelor of Science in Secondary Education once again summa cum laude.

In 2003, Michele attended Bucknell University where she joined the lab of Dr. Marie Pizzorno where her interest in viruses began, She received a Master of Science from Bucknell in 2006.

In 2006, Michele enrolled at Cornell University to obtain a Ph.D. and she joined Dr. Gary Whittaker's lab in 2007 in the Department of Microbiology & Immunology. She wishes to pursue a career focusing on public health, bio-terrorism and infectious diseases.

For everyone who believed in me and helped and supported me on this journey.

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In particular, I would like to thank Gary Whittaker for accepting me into his lab and providing the necessary guidance and support I needed during my graduate career. Gary was very patient during the years I was sick and I had few results and he always told me to do what I could do. I especially appreciate the last couple years when I would walk in his office and inform him that I had an idea and I wanted to try something and he would just grin.

I would like to thank my Special Committee members, Dr. William Brown, Dr. James Casey, and Dr. Gary Blissard for their advice and helpful suggestions.

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Finally, I'd like to acknowledge my daughters. I started college when they were in their early teens and we would spend our evenings studying and doing our homework together. It wasn't easy for them and I am forever grateful.

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Introduction

Arenavirus Classification

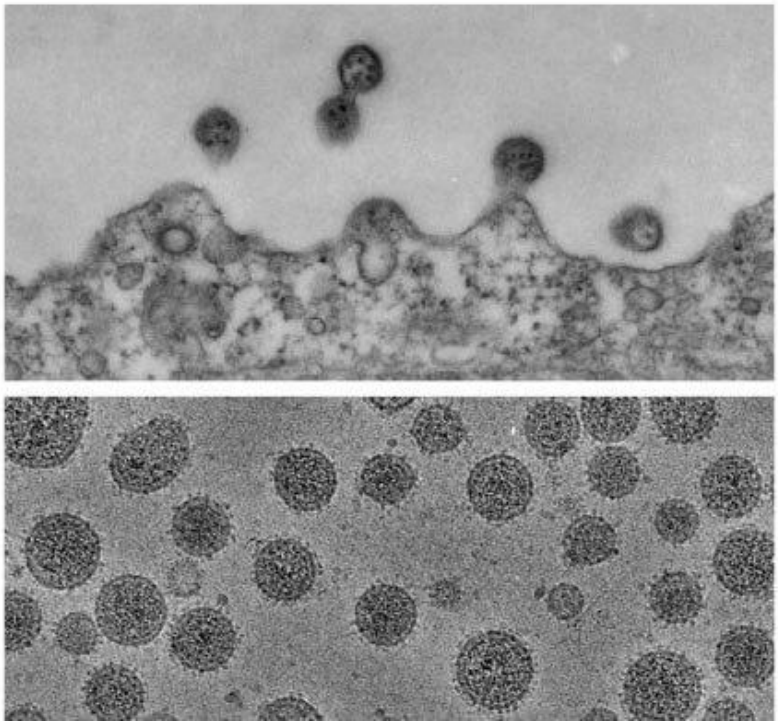
Arenaviruses belong to the family *Arenaviridae* named for the small sand-like particles located within the viral envelope (Fig. 1.1) (16, 78). The particles have since been identified as host-cell ribosomes that are incorporated into the virion during budding. Arenaviruses are currently the only virus known to package host cell ribosomes and their function has yet to be discovered (8, 53).

The *Arenaviridae* family currently contains 28 species with 24 species that are recognized by the International Committee for Taxonomy of Viruses, 8 of which cause human disease (20, 23) (http://ictvonline.org/virusTaxonomy.asp?version=2011). In 1933, Lymphocytic Choriomeningitis Virus (LCMV) was the first arenavirus that was isolated and identified as a cause of encephalitis. Since then, new arenaviruses have been discovered every few years (16).

Based on genomic sequence and phylogenetic analysis, arenaviruses have been divided into two groups: the Tacaribe serocomplex, (or New World group) with 21 species and the Lassa-lymphocytic choriomeningitis serocomplex also known as the Old World group with 7 species (Fig. 1.2). Furthermore, the New World arenaviruses are subdivided into four lineages or clades: A, B, A/B and C (15, 21, 23). Old World and some New World Clade B viruses cause viral hemorrhagic fever (VHF) and are included in the Category A Pathogen List (CDC Select Agents Regulations (42 CFR Part 73 2008)(22).

The New World group contains viruses such as Junín, Tacaribe and Machupo (JUNV, TCRV and MACV respectively) and the Old World group has Lymphocytic Choriomeningitis (LCMV) and Lassa Fever Virus (LASV) species (16).

A



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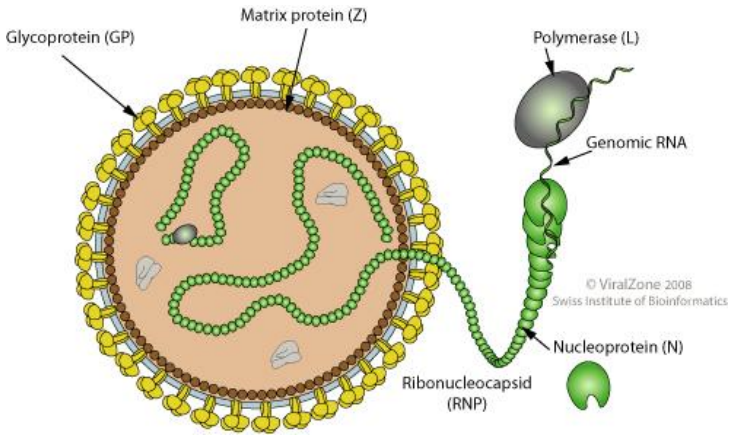

Fig. 1.1 Electron microscopy and cartoon representations of an Arenavirus. (A) Electron micrograph of arenaviruses. The dark specks within the virion are host cell ribosomes. Glycoproteins are shown dotting the surface of the viral envelope (34). (B) A cartoon representation of a virion identifying the host cell ribosomes, proteins and structure of the virus (Source: ViralZone www.expasy.ch/viralzone, Swiss Institute of Bioinformatics).

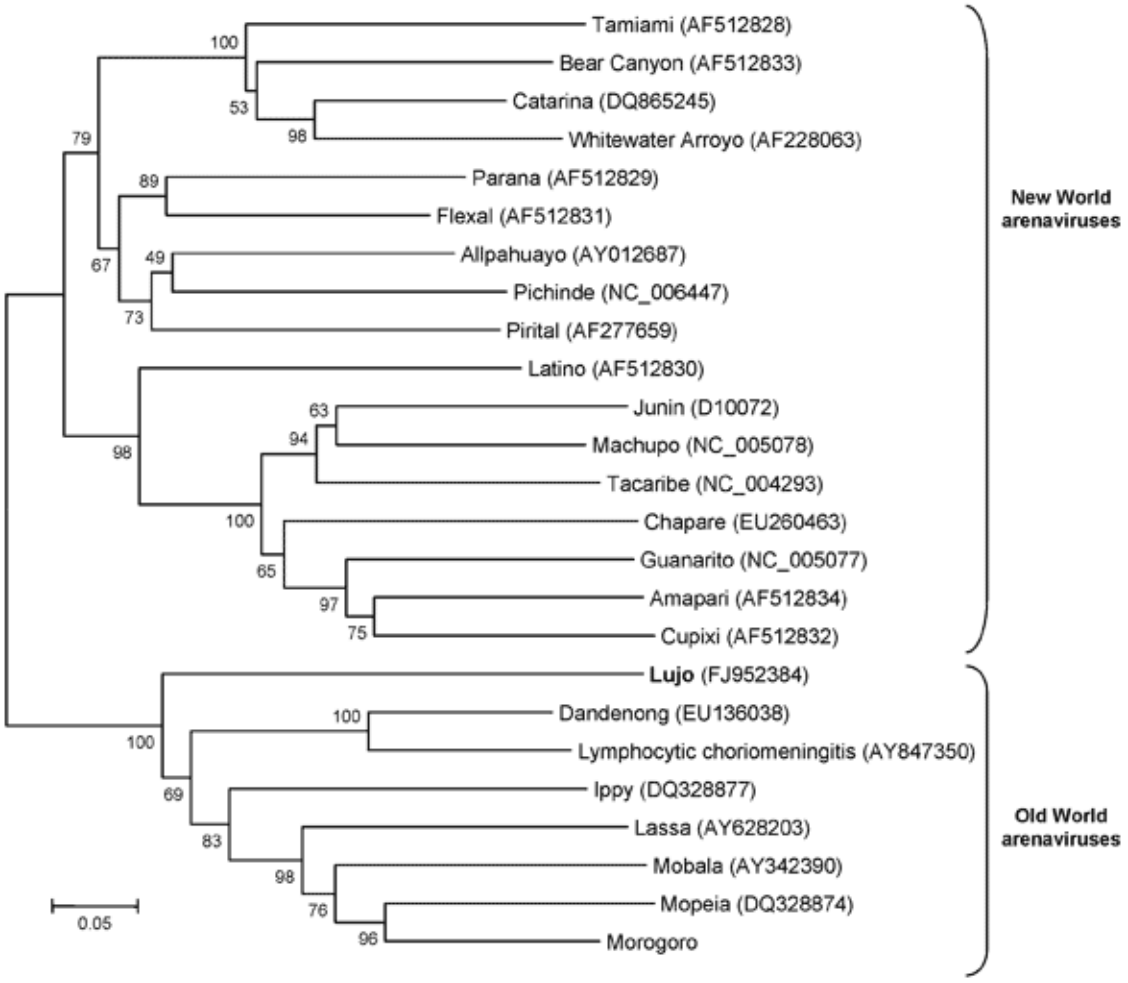

Fig. 1.2 Phylogenetic tree of Old and New World Arenaviruses.
Old and New World Arenaviruses are separated phylogenetically and geographically (67).

Pathogenesis

Rodents are the reservoirs of most arenaviruses and infections in the natural host are persistent and generally asymptomatic. In contrast, fruit bats are believed to be the natural reservoir of Tacaribe based on original virus isolation (16, 29). And more recently, several arenaviruses were discovered that infects snakes (84)

Both New World and Old World arenaviruses cause hemorrhagic disease with the New World viruses causing more severe infections. In particular, Junín virus infections, the causative agent of Argentine Hemorrhagic Fever (AHF), have an approximate 20-30% fatality rate (22, 58). Disease can manifest with fever and myalgia and progress to neurologic involvement and hemorrhaging (37). The Candid1 vaccine strain of Junín, licensed as an investigational new drug in the USA, has proven to be effective and there has been a decrease in the incidence of disease though long term studies regarding safety and efficacy of the vaccine have not been conducted (27, 31).

Infection generally occurs through exposure to infected rodent excreta, though human to human transmission is possible (22). Infection can occur by means other than direct contact and vertical transmission has been documented. LCMV is considered an emerging fetal teratogen and symptoms may include hydrocephalus, chorioretinitis and mental retardation (9). Additionally, LCMV infection has been identified in organ transplant recipients in the United States. In 2005, 3 of 4 people died after receiving organs from an infected individual. The infection was eventually traced back to a pet hamster owned by the donor (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm54d526a1.htm).

Tissue analyses have shown that epithelial cells, adrenal cortical cells, dendritic cells, hepatocytes, macrophages and monocytes can be infected by arenaviruses (58). The progression

of infection is not well understood but it is postulated that infected monocytes and macrophages may be responsible for disseminating the virus throughout the host (37, 49, 61).

Genome and Structure

Arenaviruses are enveloped bi-segmented RNA viruses that replicate in the cytoplasm of infected cells. The genome is ambisense with the positive sense RNA located at the 5' end of the sequence. The genome consists of a large (L), 7.5 kb, and a small (S), 3.5 kb, RNA segments arranged within a helical nucleocapsid. The L segment codes for the matrix (Z) protein, and the RNA-dependent-RNA-polymerase. The S segment encodes the glycoprotein precursor (GPC) and the nucleocapsid (N) protein (Fig. 1.3A) (16).

Arenaviruses have a complicated method of expressing and replicating their genome (Fig. 1.3B). The RNA-dependent-RNA-polymerase (L) is included in the virion. Once the virion undergoes fusion in an acidifed endosome, the viral genome is released into the cytoplasm. The polymerase can bind to the 3' end of the genome and transcribe the (-) sense RNA into a (+) mRNA. The mRNA is then translated via the host cell machinery and produces either the nucleoprotein or the polymerase depending on which segment (L or S) the polymerase has bound to. In order to transcribe the 5' end of the genome, a complete anti-genome is first produced. After production of the anti-genome, the viral polymerase can then bind to the 3' end and transcribe and translate proteins as just described.

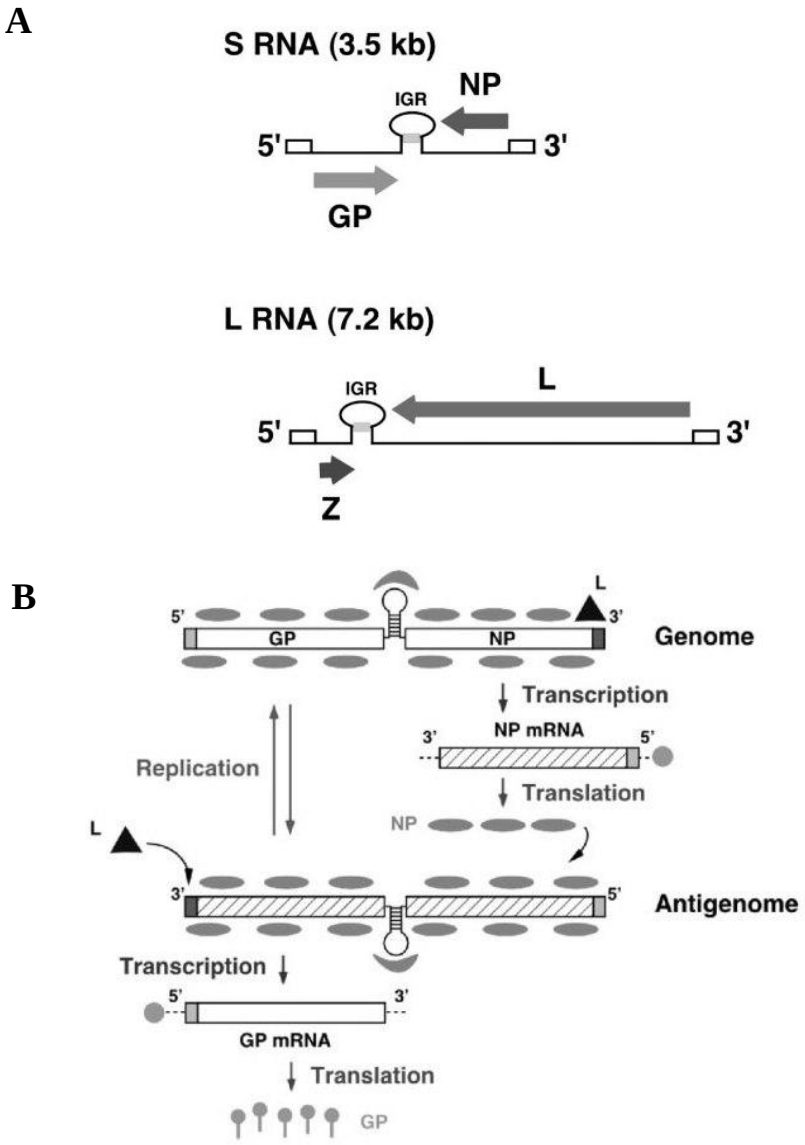

Fig. 1.3 Representation of the bipartite genome and ambisense replication strategy. (A) The bipartite viral genome is comprised of a short (S) and a long (L) segment. The S segment encodes the viral glycoprotein and nucleoprotein while the L segment encodes the matrix protein (Z) and the RNA-dependent-RNA-polymerase (L). (B) The 3' end of the genome is transcribed first by the viral polymerase, L, forming a positive stranded mRNA which is then translated to produce the protein. The 5' end of the genome must be replicated forming and anti-genome. Once this step has occurred, the viral polymerase can then bind and transcription and translation proceeds as described earlier (34).

The pleomorphic virus particles are spherical with a diameter ranging from 40-200nm. The viral envelope is studded with a glycoprotein complex composed of GP-1 and GP-2, which are the cleavage products of GPC, non-covalently bound together. The complex is arranged in trimers. A stable signal peptide (SSP) and the matrix protein associate with and are sub-adjacent to the GPC complex (Fig. 1.4) (16).

The partial crystal structure of the Machupo Virus GP-1, a Clade B virus, was recently determined (Fig. 1.5). The structure includes residues 87-257 (the complete GP1 consists of residues 59-257 (14). However, there was no electron density for the last 20 residues. Additionally, a structure showing New World Arenavirus Machupo bound to its transferrin receptor 1 (TfR1) has been solved (Fig. 1.5) (1) as has the GP-2 post-fusion structure for LCMV (42)

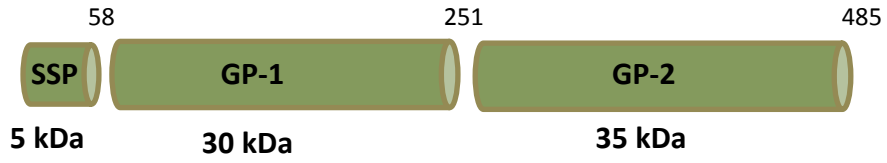

FIG. 1.4 Schematic diagram of Junin virus Glycoprotein Precursor. The glycoprotein is produced as a 485aa precursor. The 58aa stable signal peptide (SSP) is cleaved by the ER SPase after the nascent protein is translocated into the ER. The cleaved SSP associates with the C-terminus of GP-2. The GPC complex is folded and escorted out of the ER into the Golgi where GP-1 is cleaved from GP-2 by SKI-1/S1P. GP-1 binds non-covalently to GP-2 and the GPC complex is transported to the cell surface.

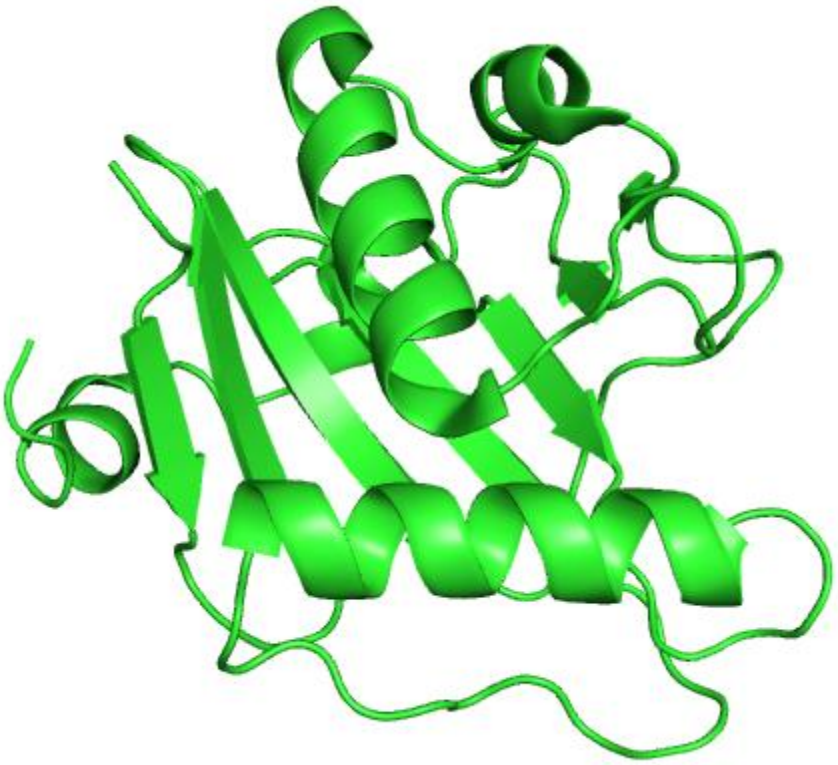

Fig 1.5 The solved crystal structure of Machupo New World Arenavirus GP-1. Machupo (MACV) GP-1 residues 87-257, were expressed, purified and crystalized (PDB code 2WFO).

Arenavirus entry

Viruses are obligate intracellular parasites that require entry into host cells to replicate their genome. In order to enter a cell, the virus must first bind with cellular surface structures which may act as attachment factors or as receptors. Binding to attachment factors, such as C-type lectins, may be via low affinity, non-specific interactions or due to electrostatic attraction of viral residues to charged cellular proteoglycans. Attachment factors may serve to concentrate virus on the cell surface to facilitate receptor binding (7, 59, 86). Conversely, receptor binding is specific and this specificity determines the host range, known as tropism (82). Some viruses, such as HIV, and HCV use multiple co-receptors to successfully enter a cell (6, 10, 33).

The arenavirus envelope is studded with glycoproteins, GP-1, GP-2, and the stable signal peptide (SSP) complexed together through ionic bonds (16). GP-1 is involved in virus binding to the receptor (13) and GP2 mediates fusion of the viral envelope with the endosomal membrane (28, 36). The cellular receptor of Old World and New World Clade C arenaviruses has been established as α-dystroglycan (17, 83). Pathogenic New World arenaviruses utilize transferrin 1 receptors (TfR1) from their natural host for binding and entry, though pathogenic strains can also enter cells using a less efficient TfR1-independent pathway (35).

TfR1 is a ubiquitous cellular receptor expressed on endothelial cells, macrophages, and activated lymphocytes and is thought to be up-regulated during arenavirus infection which allows the virus to spread and replicate more efficiently within the host. TfR1 is used by the cell to transport the iron-binding protein transferrin into the cell and regulation of TfR1 is dependent on iron levels within the cell. TfR1 associates with clathrin-coated pits and following transferrin binding is endocytosed into an early endosome. The iron binding site is separate from the viral binding site and does not appear to affect virus binding (72).

Pathogenic New World arenaviruses can effectively use human TfR1 (hTfR1) (Fig. 1.5) to infect cells which explains their ability to cause disease in humans (2). Additionally, pathogenic New World strains can efficiently use TfR1 orthologs of their natural host to gain entry into the cell. A key residue, tyrosine 211, was shown to be important for efficient use of an ortholog receptor (73) although the presence of tyrosine 211 did not guarantee its use as a receptor as evidenced by pseudovirions inability to utilize canine TfR1 (cTfR1) (Fig. 1.5). However, the feline (*Felis catus*) transferrin receptor, fTR1, possess the tyrosine 211 and is efficiently used by JUNV to enter cells Closely related orthologs such as *Mus musculus* (mTfR1) and *Rattus norvegicus* lack the tyrosine 211 and do not facilitate infection.

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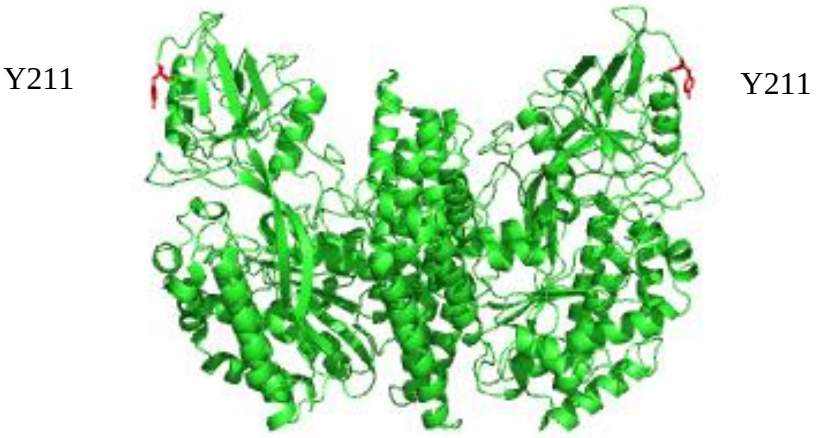

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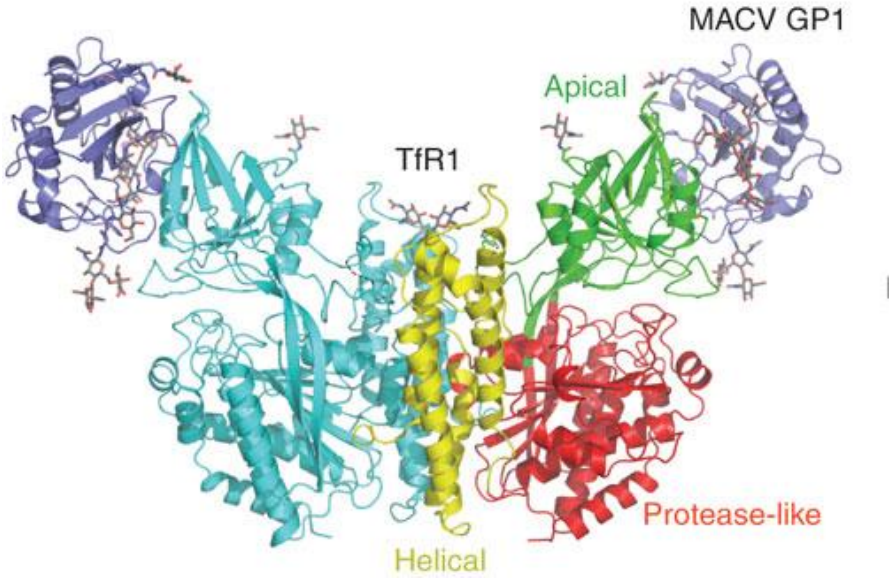

Fig. 1.6 The human transferrin receptor 1 bound to GP-1.
Pathogenic New World Arenaviruses can utilize hTfR1 to bind and enter cells. (A) hTfR1 is shown with the key residue, tyrosine 211, highlighted in red. (PDB 1CX8) (www.Pymol.org) . (B) The GP-1 of Machupo binds to TfR1 via tyrosine 211. Once bound, the receptor is then endocytosed (1).

Entry is dependent on viral binding to its receptor(s). Once bound to a receptor, enveloped viruses use multiple methods to enter cells. The most common method involves endocytosis of virus via clathrin-coated pits. Upon receptor binding, clathrin-coat components are recruited and a coated pit forms underneath the virus bound receptor. The pit invaginates and dynamin pinches off the neck producing a clathrin-coated vesicle which is then uncoated and transported to an endosome. The New World virus Junín enters cells using this route (57, 77).

Some Old World arenaviruses have been shown to use a recently identified endocytic pathway. This pathway is clathrin and caveolin independent but requires membrane cholesterol. The virus is endocytosed in a smooth walled vesicle. The vesicle bypasses early endosomes and is delivered to a late endosome where membrane fusion occurs and the virus penetrates into the cytosol (71, 76, 77).

Both entry pathways utilized by arenaviruses require the presence of fusion proteins on the viral envelope. Viral fusion proteins undergo a conformational change that is triggered by maturation and acidification of an endosome. There are three classes of fusion proteins based predominately on secondary structure. Class I fusion proteins, such as influenza HA, exists as trimers and are highly α-helical with the fusion peptide located internally or at the N-terminus. Class II fusion proteins such as Semliki Forest Virus (SFV) E1 exist as dimerized β-sheets that trimerize during fusion activity. The fusion peptide exists as an internal loop (41, 46). Vesicular stomatitis virus (VSV) is considered to be a Class III fusion protein and is predominately a β-sheet. It exists as a homotrimer which can undergo reversible conformation changes when exposed to low pH (25). After fusion occurs, the virus capsid penetrates into the cytoplasm of the cell releasing the viral genome (82). Arenaviral GP-2 has a series of alpha-helical heptad repeats and is considered a Class 1 fusion protein such as influenza (32). Experiments with

LCMV identified irreversible conformational changes upon GPC exposure to low pH. GP-1 was shown to dissociate from GP-2 at pH 5. Use of conformational specific GP-1 antibodies indicated a conformational change occurs at pH 5 as evidenced by reduced binding to GP-1. Conversely, GP-2 antibodies showed a 4-fold greater binding at low pH treatment. The N-terminal hydrophobic amino acids W264, G277, Y278 and L280 of LASV GP-2 were shown to be involved in fusion (28, 48).

It is presently unknown if arenaviruses utilize attachment factors or co-receptors in the initial binding steps. It is predicted that arenaviruses, such as Junín and Tacaribe, have asparagine-linked (N-linked) glycosylation sites (4). N-linked glycosylation sites have been shown to play an important role in SARS-coronavirus entry. SARS uses angiotensin-converting enzyme 2 (ACE2) as the receptor while DC/L-SIGN are used as alternative receptors independently of ACE2. DC/L-SIGN recognize N-linked glycans and treatment of viral particles with Endo H reduces DC/L-SIGN related infectivity (40).

Glycoprotein post-translational processing

The envelope glycoprotein precursor (GPC) polypeptide is cleaved into three distinct subunits: the stable signal peptide (SSP), the receptor binding (GP-1) and the fusion (GP-2) subunits. SSP guides the nascent protein into the lumen of the endoplasmic reticulum (ER) where it is cleaved by the signal peptidase. The SSP remains associated with the GP-1/GP-2 complex. The 58 amino acid long SSP subunit is unusually lengthy since SSPs generally range between 13 and 36 amino acids long (88).

The SSP is myristoylated at the N-terminus penultimate glycine and the GP-1/GP-2 complex is glycosylated. A myristoylation motif, G-X_3-S/T, is present at the SSP N-terminus of all known arenaviruses. Myristic acid is a hydrophobic 14-carbon saturated fatty acid that is

added co-translationally (93). Arenaviral SSPs have been shown to be myristoylated. Mutation of the SSP N-terminal glycine to an alanine has been shown in various studies to prevent myristoylation but did not affect assembly or transport of the GPC complex to the cell surface. However, cell-cell fusion was reduced when compared to wild type suggesting that myristoylation is required for efficient membrane fusion (79, 81, 93). Additionally, myristic acid analogs have been shown to inhibit Junín replication. Inhibition of protein myristoylation did not significantly affect intracellular transport or expression of glycoproteins on the cell surface (24).

Many viral glycoproteins are glycosylated and viruses utilize the host cell machinery to accomplish this process (12, 40, 52, 75, 87). Protein glycosylation is a post-translational modification that occurs within the lumen of the ER. In particular, N-linked glycosylation involves addition of a high mannose core structure to the amide nitrogen of asparagine located within an Asparagine-X-Serine/Threonine motif with X being any residue but proline. The mannose core is further modified by trimming and processing of glucose and mannose from the core. Additional sugars such as fucose, galactose, GalNAc and GlcNAc may be added as the protein progresses through the ER and Golgi resulting in a complex hybrid structure (85, 87). N-linked glycosylation can facilitate correct folding and assembly which allows the protein to traffic from the ER through the Golgi. Additions or deletions of glycosylation sites can alter the final conformation of the protein thereby interfering with transit or functionality such as abolishing the protein's ability to bind a receptor (39, 50, 54). Many misfolded proteins are retained in the ER. Calnexin and calreticulin normally bind proteins and aid in proper folding. Deglycosylating the proteins interferes with this chaperone activity. However, glucosyltransferase (GT) recognizes misfolded proteins and generally prevents their exit from

the ER. GT recruits calnexin and calreticulin to the protein which is refolded. (26, 50, 60, 65). Misfolded VSV has been shown to aggregate in the ER when glycosylation is inhibited (51). However, VSV mutants can achieve proper folding when grown at a lower temperature. It is thought that low temperature conditions allow the protein to reside in the ER for a longer period of time and this extended time frame allows for correct folding (38).

The levels of glycosylation vary across glycoproteins. Junín has 4 predicted N-linked glycosylation sites on GP-1 while HIV glycoprotein, gp120, has about 24 glycosylation sites many of which are highly conserved (52).

Experimentally, glycosylation sites can be removed biochemically by the addition of glocosidase enzymes such as Endoglycosidase H or PNGase F (Endo H) (26, 85). The molecular mass of the JUNV GPC complex is between 65-70 kDa. Approximate values for individual subunits were: SSP-5kDa, GP-1-30kDa and GP-2-35 kDa. Deglycosylation with PNGase F resulted in a GP-1 of 25kDa and a GP-2 of 28 kDa indicating that JUNV GPC is glycosylated (93). Additionally, tunicamycin, a glycosylation inhibitor, has been shown to interfere with Sindbis, vesicular stomatitis, herpes simplex and Junín virus infectivity (45, 51, 66). Influenza hemagglutinin (HA) has N-linked glycosylation sites which are critical for glycoprotein folding and trafficking to the plasma membrane (26). HA glycans located near the receptor binding domain are important for affinity and receptor binding itself (47).

Also, glycosylation sites can be added to viral glycoproteins by using specific primers to mutate an existing residue or by inserting an additional residue into the sequence. Addition of N-linked glycosylation sites on VSV resulted in the protein being expressed on the cell surface; however, the efficiency was reduced when compared to wild type and for some mutants because the additional site caused inhibitory effects on transport (56).

Alterations in glycosylation can be beneficial. Loss of glycosylation near an Influenza HA cleavage site allowed for more efficient cleavage and activation of the HA and enhanced virulence (74). Removal of N-linked glycosylation alters monoclonal antibody recognition of antigenic sites on VSV possibly due to altered conformation and masking of the site (39).

The glycosylation state of viral proteins is important for host cell immune system interactions. Glycoproteins are the major target of neutralizing antibodies (70). Carbohydrate recognition molecules can recognize glycosylation patterns on viruses and aid in clearance of the pathogen from the host. Including for example mannose binding lectin (MBL) and C-type lectins (87). MBL interacts with all HIV gp120 strains and has a slight neutralization effect on the virus (44). Additionally, neutralizing antibody against HIV gp120 glycosylation mutants had little effect on wild type glycoprotein suggesting that epitopes had been masked by conformational folding (70).

Topology

Arenaviruses have two hydrophobic domains, h1 and h2, located within the SSP subunit. These domains vary slightly between arenaviruses and also according to the prediction program used. Either domain promotes insertion of GPC into the ER membrane (30, 79, 81). However, the C-terminal h2 is more efficient possibly due to its proximity to the signal peptidase cleavage site. Both hydrophobic domains are required for GP-1/GP-2 cleavage and resultant plasma membrane expression (81).

Once GP-1 and GP-2 are cleaved, they form a non-covalent bond with GP-1 forming a cap like structure on the GP-2 stalk like structure (Fig. 1.7). The SSP remains associated with GP-2 and the membrane topology of SSP is considered to be bitopic with both the N and C terminus extending into the cytoplasm (Fig. 1.7). Both hydrophobic domains are 15 amino acids

long with an 8 amino acid ectodomain inserted between them (4) The hydrophobic domain of signal sequences is generally between 10-15 amino acids long and is thought to adopt an extended chain structure which is partly α-helical and has the ability to perturb the membrane creating a local defect (64, 88). Only eight amino acids are required to span the plasma membrane as an extended chain while 20 are required for an α-helical structure (88). The two hydrophobic domains are separated by a lysine at residue 33 which is located within the ectodomain (Fig. 1.7).

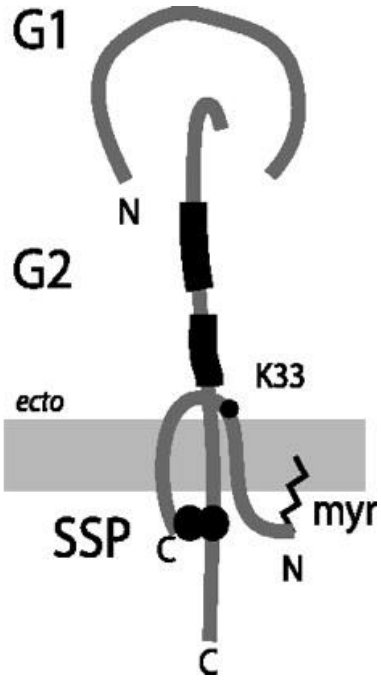

Fig.1.7 Representational drawing of the Junín GPC complex. Once the stable signal peptide is cleaved, it associates with the C terminus of GP-2. The SSP passes through the membrane twice and the myristoylated N terminus is embedded in the membrane. GP-1 (G1) is non-covalently bound to GP-2(G2) after they are cleaved in the Golgi. The two hydrophobic domains are seen as black rectangles on G2. The lysine residue at position 33, which is critical for fusion, is shown as K33(63).

Exocytosis

After cleavage by SPase I, the SSP subunit continues to associate with the GP-1GP-2 precursor and is required for transport of GP-1/GP-2 from the ER. SSP is thought to interfere with dibasic ER localization signals located within the cytoplasmic tail domain (CTD) of GP-2 thereby allowing the GP-1/GP-2 complex to successfully exit the ER lumen (4, 89, 90). Upon exiting the ER, the GP-1/GP-2 complex is proteolytically cleaved by SKI-1/S1P in the Golgi producing the individual GP-1 and GP-2 subunits. GP-1 associates with SSP/GP-2 and is thought to act as a chaperone for processing and localization to the plasma membrane (43). Three residues (KRR) located at the C-terminal end of GP-2 are critical for SKI-1/S1P cleavage and their deletion blocks cleavage. A furin cleavage site (RRKR) can substitute and effectively replace the SKI-1/S1P cleavage site resulting in infectious virus that is lower in efficiency than wild type (5). After GP-1/GP-2 cleavage, SSP associates with the ectodomain and cytoplasmic tail of GP-2. GP-2 has a high affinity zinc binding domain in the CTD of GP-2 which is thought to incorporate SSP into the GPC complex (11, 91). The SSP/GP-2 complex is involved in fusion of the viral envelope and cellular membrane induced by the lower pH found within the endosome (92). The positively charged K33 found within the ectodomain of SSP has been shown to be critical for membrane fusion (4, 79).

Junín SSP was replaced with the chimeric CD4 signal peptide to determine if the signal peptide had a function other than targeting the nascent GPC to the ER. The resultant mutant was assembled and transported to the cell surface however the construct was unable to undergo cell-cell fusion when exposed to low pH suggesting that SSP has additional functions beyond polypeptide targeting (93).

Assembly

The Junín-GPC complex is not found within detergent resistant lipid rafts on the plasma membrane; however, the GPC complex clusters on the plasma membrane within discrete microdomains ranging from 120-160 nm in diameter. GPC myristoylation has no effect on GPC microdomain assembly and Z matrix proteins are not found to colocalize within the domain (3). The Z protein interacts with the N, nucleocapsid protein, and this interaction is required for recruitment of nucleocapsids to the plasma membrane and assembly of nucleocapsids and the GPC complex into budding particles (19, 68). Z is myristoylated and removal of the N terminal myristic acid inhibits budding and significantly impacts the Z-GPC interaction at the plasma membrane (18, 69). A late domain motif on Z has been shown to interact with Tsg101, a cellular protein involved in vacuolar sorting and part of the multi-vesicular body budding pathway, suggesting the interaction is involved in viral budding from the plasma membrane(68).

Additional viruses have proteins with structural and functional similarities to SSP. Semliki Forest and Sindbis viruses have a 6K protein that has been shown to be necessary for viral budding 6K has a size range between 55-58 amino acids with a hydrophilic N-terminus and two hydrophobic segments that are separated by 1-2 basic residues (55, 62). Additionally, a weakly conserved domain within SSP is similar to one found to be critical for paramyxovirus budding. Mutation of P12 within SSP affects formation of virus like particles (VLP) (79).

The mature LASV GP-1/GP-2 complex forms trimers that are inserted into the viral envelope. Cholesterol depletion has no effect on trimer formation but impairs infectivity. The GP complex and M protein are located and bud from detergent soluble regions of the plasma membrane (80).

Summary

The main objective of this thesis is to characterize entry and pathogenesis of Junín Arenavirus. Chapter 2 begins with identification of DC-SIGN and L-SIGN as potential receptors and shows that immature dendritic cells are *trans*-infected and can transmit virus to other cell types. Chapter 3 identifies LSECtin as a primary receptor for Junín virus. Chapter 4 explores the N-linked glycosylation sites on Junín GP-1 and identifies them as being critical for virus structure and function. Finally, Chapter 5 will summarize my research findings.

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Chapter 2

Junín Arenavirus *in-vitro* infection is mediated by *trans*-infection of immature Dendritic Cells.

Introduction

The family *Arenaviridae* is divided into two major complexes, the New World and the Old World complex (7, 14). Seven of the known arenaviruses can cause viral hemorrhagic fever (VHF) in humans, with five of them, namely Junín, Guanarito, Machupo, Chapare, and Sabiá, belonging to the phylogenetic clade B of New World arenaviruses (8, 9, 13, 18). Rodents are the known reservoir of most arenaviruses and infection generally occurs after exposure to infected rodent excreta. Junín infection initially shows non-specific flu-like symptoms. As the disease progresses, hemorrhagic and neurological complications may occur with mortality rates ranging from 20-30% (Marty et al., 2006; Peters, 2002).

Arenaviruses are enveloped, and contain two segments of ambisense single-stranded RNA. A large segment (L) encodes the matrix protein (Z) and the RNA-dependent-RNA-polymerase (RdRP). The small segment (S) encodes the nucleocapsid-associated protein, (NP) and the glycoprotein precursor (GPC). The GPC precursor is proteolytically processed during virus assembly to produce GP-1, which is involved in receptor binding and GP-2, a membrane-anchored domain necessary for fusion (6, 19, 55, 56). An additional cleavage product of GPC, the stable signal peptide (SSP), associates with GP-1 and GP-2 and is required for efficient trafficking to the plasma membrane and subsequent fusion of the viral envelope with the cellular membrane of infected cells (2, 71-73).

Entry of human pathogenic arenaviruses into host cells has been studied extensively (61), in particular with regard to receptor utilization. Several members of the Old World complex and the New World clade C complex use α-dystroglycan as a cellular receptor (10, 64). New World Clade B arenaviruses utilize the transferrin receptor (TfR1) from their natural host (1) and it has been shown that all pathogenic members of the New World group use human TfR1 as a receptor

to infect human cells (58, 59). However, a TfR1-independent entry pathway has also been suggested for members of this clade: Experiments using siRNA knockdown of TfR1 in mouse cells resulted in no reduction in titer relative to the controls indicating that murine TfR1 plays no role in entry into these cells (15, 27) implying that multiple receptors exist.

The TfR1 receptor is found on the basolateral surface of the human airway epithelium (20, 53) which is inaccessible to inhaled viral particles. In addition to having a primary receptor, it is possible that arenaviruses may use non-specific receptors or attachment factors during entry. DC-SIGN(+) dendritic cells have been shown to be present in sub-epithelia tissues of the respiratory tract including the bronchi, mouth and trachea (17). Dendritic cells are antigen presenting cells (APCs) that monitor tissues for the presence of pathogens (67). Upon capture of a pathogen, DCs can express co-stimulatory signals and will migrate from the peripheral tissue to a draining lymph node where it stimulates T-cells (60).

Dendritic cells are recruited to airway mucosa (50) upon inflammatory stimulation induced by the presence of a pathogen (49, 51). Work with measles virus (MV) suggests that recruited DC-SIGN(+) DCs bind MV and transport the inhaled virus to lymphoid tissues (17, 32, 38). Influenza A has also been found in the draining lymph nodes (35) indicating that dendritic cells can migrate to draining lymph nodes upon presentation of viral antigen in the lungs (42).

DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) is a C-type lectin found on dendritic cells and subsets of macrophages (16, 28). Its homolog DC-SIGNR also known as L-SIGN (Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin) is found on liver endothelial cells and lymph nodes (5). Both lectins interact with pathogens through their carbohydrate recognition domain (CRD) to bind and internalize pathogens for destruction (25, 54). Various viruses such as dengue, ebola, HCMV, HCV, HIV-1,

measles, and SARS-CoV have subverted this system and use the lectins to infect host cells bearing these lectins (3, 17, 29, 34, 57, 66, 70). Many of these viruses use dendritic cells as Trojan horses and the now trans-infected DC carries the virus through the lymphatic system to susceptible cells in lymphoid tissues (3, 17, 37, 43, 45, 48, 70). However, the role of these antigen presenting cells during New World arenavirus infection remains unexplored.

DC- and L-SIGN interact via their C-terminal carbohydrate recognition domain (CRD) with mannose residues on viral glycoproteins (25, 54). Viruses including HCV, SARS-CoV, HIV-1, HCMV, dengue and ebola bind to these lectins via their envelope glycoproteins (3, 12, 28, 34, 36, 57, 66). Motifs located within the cytoplasmic domain of the lectin are involved in the endocytosis and recycling of the lectin back to the plasma membrane (23, 44, 67), and may also play important roles in signaling events in infected cells subsequent to virus binding. Studies have shown that Junín virus has mannosylated residues (33) and based on this observation and previous studies identifying DC/L-SIGN binding to mannose residues on viral glycoproteins, I investigated the roles of DC-SIGN and dendritic cells on Junín virus entry and infection. I used pseudotyped retroviral particles carrying functional GPC (JUNVpp). My results show that JUNVpp can interact with DC-SIGN and that dendritic cells become *trans*-infected thereby indicating an important role in viral pathogenesis.

Methods

Cell Culture

HEK 293T/17 (ATCC CRL-11268), NIH/3T3 cells (ATCC CCL 1658), and 3T3-derived DC-SIGN and L-SIGN cells (provided by the NIAID AIDS Research and Reference Reagent Program) were grown in DMEM (Cellgro) supplemented with 10% FBS, penicillin/streptomycin and were supplemented with HEPES (20 μM). Human monocyte derived dendritic cells (Astarte Biologics) were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, and 1ng/mL each of GM-CSF and IL4 (BD Bioscience). Human hepatic sinusoidal endothelial cells (HHSECs) (ScienCell) were grown in complete endothelial cell medium (ScienCell 1001) and cultured in flasks pretreated with fibronectin (ScienCell 8248). In all cases, cell cultures were grown at 37°C in 5% $CO_{2.}$

Reagents and antibodies

Mannan and mannose were obtained from Sigma. Anti-Junín monoclonal antibody MAb GB03-BE08 was kindly donated by Dr. A. Sanchez (Centers for Disease Control, Atlanta GA, USA). Anti-DC-SIGN and anti-DC/L-SIGN MAbs respectively (9E9A8, 14EG7) were obtained from the NIAID AIDS Research and Reference Reagent Program or from Santa Cruz Biotechnology Inc. Monoclonal anti-DC-SIGN (MAB161) and anti-L-SIGN (MAB162) used for blocking were purchased from R&D Systems. Polyclonal anti-DC-SIGN (ab97526) anti-L-SIGN (ab58603), anti-Lamp-1 (ab24170) and anti-EEA1 (ab2900) were obtained from Abcam. Polyclonal LSECtin (sc-70177) was purchased from Santa Cruz. Alexa Fluor secondary antibodies, Alexa Fluor 568-conjugated transferrin, and Prolong Gold Antifade with Dapi were obtained from Molecular Probes. The anti-VSV antibody P5D4 was obtained from Sigma.

Plasmids

Constructs pcDNA3.1-DC-SIGN and pcDNA3.1-L-SIGN (NIAID AIDS Research and Reference Reagent Program) expressing the human (*H. sapiens*) receptors were used to transiently express C-type lectins using Turbofect as recommended by the manufacturer. pcDNA3.1 plasmids expressing the human transferrin receptor (TfR1) was provided by Dr. Colin Parrish (James A. Baker Institute for Animal Health, Cornell University). pcDNA3.1 (-) (Invitrogen) and pEGFP C1 (Clontech) were used as controls. A codon-optimized version of GPC was synthesized using the sequence of Junín virus strain IV4454, GenBank: DQ272266.3 (GeneArt) and subsequently subcloned into the pcDNA3.1 expression plasmid (Invitrogen).

Production of Pseudotyped Virions

Pseudotyped virions were produced as described previously (4). 293T cells were cotransfected at a ratio of 1:1:1 with an MLV-based transfer vector encoding luciferase, an MLV Gag-Pol packaging construct, and an envelope glycoprotein expressing vector (pcDNA3.1-JUNV GPC) by using Turbofect (Fermentas), as recommended by the manufacturer. Cells were incubated at 37°C for 48 h, and after harvesting the supernatants were filtered through 0.45-µm pore sized membranes (Sarstedt).

Site-directed mutagenesis Plasmids pcDNA3.1 DC-SIGN-LL/AA (DAA) and L-SIGN-LL/AA (LAA) were generated by using a Quikchange site-directed mutagenesis kit (Stratagene) to mutate the di-leucine internalization motif to di-alanine. All constructs were sequenced to confirm mutagenesis.

C-type lectin expression

3T3 cells were transfected separately with WT human DC-SIGN and L-SIGN and mutant DC- and L-SIGN using Lipofectamine 2000 as recommended by the manufacturer. Twenty-four hours after transfection, the cells were treated with EZ-link sulfo-NHS-SS biotin (ThermoScientific) to label surface proteins. The cells were washed with glycine and lysed with TBS/NP40 containing Complete Protease Inhibitor (Roche). The biotinylated surface proteins were separated from the lysate using Pierce Streptavidin Agarose Resin (Thermo Scientific) and constant agitation at 4°C overnight. Samples of cellular lysate were used to detect intracellular lectin. Surface proteins and intracellular protein lysates were separated by SDS-PAGE and immunoblotted on nitrocellulose. DC-SIGN and L-SIGN WT and mutants were detected using polyclonal anti-DC-SIGN (ab97526) and L-SIGN (ab58603) and HRP-conjugated secondary antibodies. Blots were visualized using Pierce ECL Substrate (ThermoScientific) and imaged and quantified on a Fuji LAS-3000.

Transduction of 3T3 cells

3T3 cells plated in 12 well plates were first co-transfected (Turbofect) with 500 ng of plasmid coding for transferrin receptor (pcDNA3.1-TfR1,) in combination with 500 ng pcDNA3.1-DC-SIGN or pcDNA 3.1-L-SIGN, with pcDNA3.1 as a control. In a different set of experiments, 3T3 cells were transfected with pcDNA3.1-TfR1, pcDNA3.1-DC-SIGN(LL/AA), pcDNA3.1-L-SIGN(LL/AA), pcDNA3.1-DC-SIGN, pcDNA 3.1-L-SIGN, or pcDNA3.1 (-) as a control. Twenty-four h after transfection, transfected cells were plated in 48 well plates. Transduction with the pseudotyped virions was performed the following day at 37°C for a minimum of 4 h. The inoculum was removed and luciferase activity was measured 48 h post-transduction using a

Luciferase Assay Kit (Promega) and light emission measured by using a Glomax 20/20 luminometer (Promega).

Transduction of dendritic cells

Immature dendritic cells (iDCs) were cultured at 2 x 10^5 cells per cm^2 on poly-L-lysine coated plates. Cells were transduced with JUNVpp for a maximum of 4 h. The cells were washed with sterile PBS to remove unbound virus and allowed to incubate for a total of 48 h before luciferase activity was measured using a Luciferase Assay Kit (Promega) and light emission measured by using a Glomax 20/20 luminometer (Promega).

Carbohydrate and antibody blocking assays of dendritic cell receptors

iDCs were plated onto poly-L-lysine coated dishes. Plated cells were pre-treated with 50 or 100 μg/mL of mannose, mannan or anti-DC-SIGN for a minimum of 1 hour. After pre-treatment, JUNVpp was added in the presence of the blocking agent and incubated for 2-4 h. Cells were washed with PBS to remove unbound virus and then re-fed with additional blocking agent. Cells were harvested after 48 h and luciferase activity was measured as detailed previously.

Colocalization assay of JUNVpp with cellular receptors and intracellular compartments

iDCs were seeded onto poly-L-lysine coated chamber slides and transduced with JUNVpp. Cells were fixed at 4 and 24 hpi. Fifteen minutes prior to fixation, select wells were treated with Alexa Fluor 568-conjugated transferrin at 37^{o}C (Molecular Probes). Cells were stained with antibodies against JUNV-GPC, LAMP-1, EEA-1, DC-SIGN, and L-SIGN. Fixed slides were imaged with a Leica TCS SP5 Confocal Microscope.

Dendritic cell mediated transmission assay

iDCs were incubated for 3-4 h with JUNVpp. Unbound virus was removed by washing the cells three times with sterile PBS. The DCs were overlaid onto confluent HHSECs, Veros or NIH 3T3 cells overnight to allow for pseudovirus transfer. The target cells were washed the following day. Transfer and transduction of target cells was quantified by measuring luciferase activity of the cellular lysate 48 h later.

Pathogenesis Model

The Pathogenesis model was drawn using Illustrator (CS4, Adobe).

Statistical Methods

Graphs and statistical analyses were produced using GraphPad Prism 5. One-way ANOVA analyses included a Tukey post-test to compare between groups. T-tests were unpaired and two-tailed. All significance values were calculated at $p < 0.05$. Error bars represented the standard deviation of the mean. All pseudovirus experiments were conducted a minimum of three separate times using duplicate plates for each separate experiment. Three western blots from multiple experiments measuring surface expression of DC-SIGN and L-SIGN WT and DC-and L-SIGN (LL/AA) mutants were analyzed using the Quantify feature in the Fujifilm Image Reader software.

Results

Junín pseudovirus particles transduce cells transiently expressing DC or L-SIGN.

To study the possibility that Junín interacts with DC/L-SIGN through the glycoprotein complex, I used murine leukemia virus particles pseudotyped with JUNV GPC (JUNVpp), according to a previously described protocol (4) allowing us to effectively avoid down-stream viral events.

Relatively non-susceptible mouse 3T3 cell lines were transfected with plasmid vectors containing DC-SIGN, L-SIGN, TfR1 or a GFP control vector. Cells were then transduced with equal amounts of JUNVpp and infectivity was measured 48 h after transduction by luciferase activity of cellular lysates (Fig. 2.1). DC-SIGN, L-SIGN and TfR1 expressing 3T3 cells were susceptible to JUNVpp infection, with L-SIGN and TfR1 expressing cells being more conducive to transduction. Control 3T3 cells expressing GFP did mediate low levels of JUNVpp entry through an unknown mechanism as described previously. There was no statistical difference between columns but there was an upward trend between WT 3T3 cells and those that transiently expressed a human receptor (Fig. 2.1). These data suggest that DC-SIGN, L-SIGN, and TfR1 promote transduction of 3T3 cells with JUNVpp.

Expression of DC-SIGN, L-SIGN or TfR1 allows transduction of mouse 3T3 cells independent of each other.

I next examined whether co-expression of a C-type lectin and human TfR1 would have a combinatorial effect on transduction levels. 3T3 cells were transfected with DC-SIGN or L-SIGN in combination with TfR1, and transduced with JUNVpp (Fig. 2.2). As shown in Fig. 2.2, the combination of TfR1 with either DC-SIGN or L-SIGN produced substantially higher levels of transduction than either receptor expressed alone.

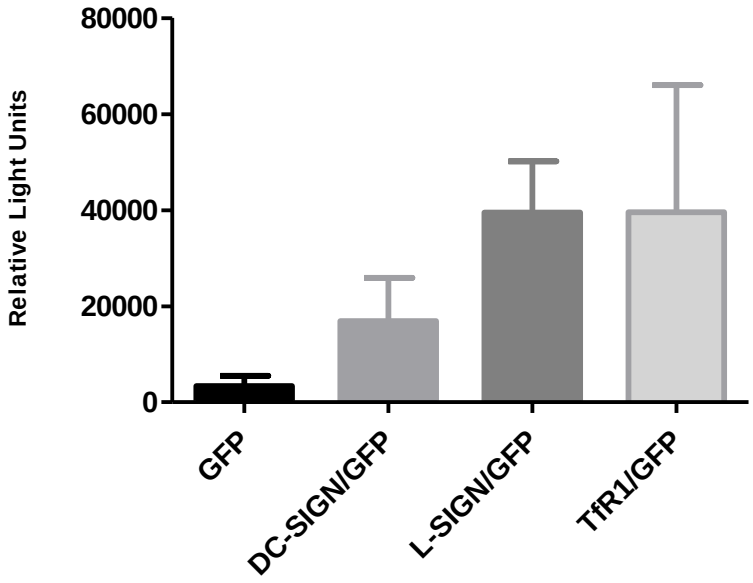

Fig. 2.1 JUNVpp can transduce cells transiently expressing DC/L-SIGN. NIH 3T3 cells were transfected with equal amounts of DC-SIGN, L-SIGN, TfR1 or a GFP expressing vector. Cells were transduced for 48 h and infectivity was measured as relative light units produced from luciferase activity using a Glomax 20/20 luminometer. The data is the results of 3 independent experiments. There is no significant difference between columns. (Experiments conducted by Dr. Sandrine Belouzard).

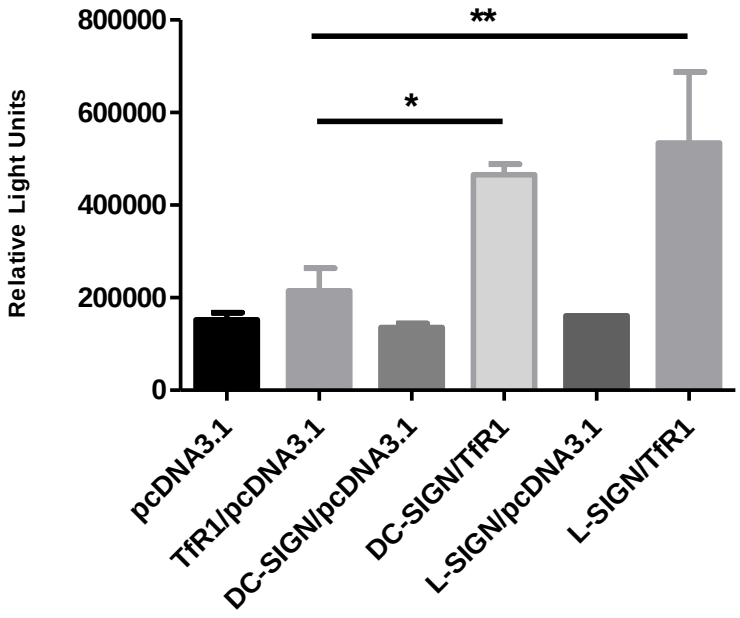

Fig. 2.2 Expression of DC-SIGN or L-SIGN in conjunction with TfR1 significantly enhanced transduction of 3T3 cells. 3T3 cells were doubly transfected with equivalent amounts of DC-SIGN or L-SIGN and TfR1. Transduced cells were lysed 48 h post-transduction and luciferase activity was measured. The data is the results of 3 independent experiments. Data is considered significant if $p < 0.05$. * $p < 0.05$. ** $p < 0.01$

Transduction of mouse cells expressing endocytosis-deficient mutants of DC-SIGN and L-SIGN.

Previous studies have identified a di-leucine motif (LL) within the C-terminal domain of DC-SIGN as being necessary for endocytosis of the receptor (24, 44, 47). An identical motif is found in L-SIGN and mutation of the LL motif is known to block endocytosis (47). To investigate the role of DC-SIGN and L-SIGN internalization for JUNV pseudoparticle entry, the leucines in the LL motif were both mutated to alanines (AA) in both DC- and L-SIGN, to create the mutants DAA and LAA respectively. Both WT and mutant DC-SIGN and L-SIGN were expressed in 3T3 cells and analyzed by cell surface biotinylation and Western Blot. Both WT and mutants were efficiently expressed on the cell surface, with LAA expressed at higher levels compared to wild type (Fig. 2.3A). Cellular lysates were also examined and similar amounts of WT and mutant receptor were present (data not shown), suggesting that any differences in cell surface expression were due to differences in trafficking and not due to alterations in protein expression.

Next, I expressed the WT and mutant C-type lectins to determine if there was an effect on JUNVpp transduction. As shown in Figure 2.3B, there was no significant reduction in transduction between WT DC-SIGN and L-SIGN and their respective endocytosis-deficient mutants, DAA and LAA. However, there was a significant decrease in transduction between TfR1 and DAA/TfR1 as compared to TfR1 and DC-SIGN/TfR1. However, there was not a significant difference between DC-SIGN/TfR1 and DAA/TfR1 and also between L-SIGN/TfR1 and LAA/TfR1.

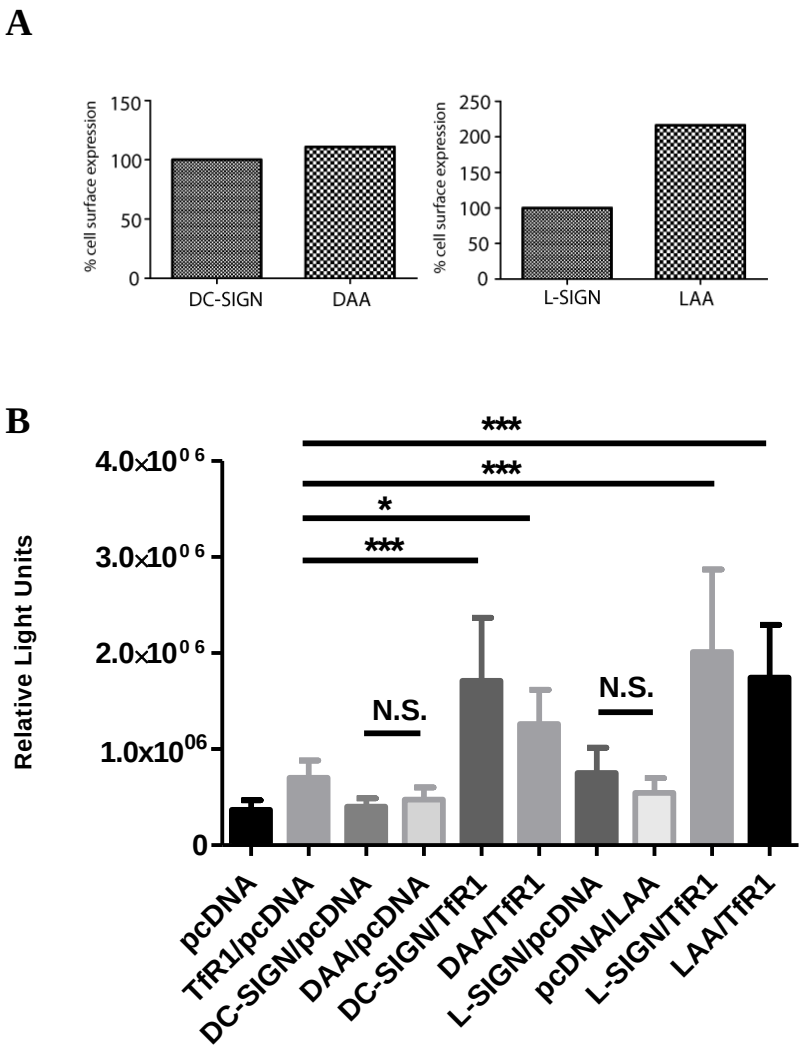

Fig. 2.3 JUNVpp can transduce cells expressing endocytosis deficient DC-SIGN and L-SIGN. (A) Endocytosis deficient DC-SIGN (DAA) and L-SIGN (LAA) expressed on 3T3 cells were biotinylated and separated by SDS-PAGE and immunoblotting. The blots were imaged and quantified with a Fuji Las 300. The data represent an average of three separate experiments. DC-SIGN and L-SIGN were normalized to 100%. (B) DC-SIGN and L-SIGN along with their respective mutants DAA and LAA were transfected into 3T3 cells and transduced with JUNVpp. The cells were lysed 48 h post-transduction and infectivity was measured as relative light units produced by luciferase activity. Data are results of a minimum of three independent experiments. N.S. non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

JUNVpp does not transduce iDCs and pretreatment with mannan or antibodies has no effect on transduction.

Dendritic cells have been considered to be infected with Junín virus based on histology and immunofluorescent assays (22, 30, 40, 41). To assess the role of dendritic cells and the DC-SIGN receptor, I used a pseudovirus system expressing the JUNV-GPC to transduce immature human dendritic cells. Transduction levels were low for all transduced iDCs and similar to the background of untreated iDCs (Fig. 2.4A). To further assess the role of DC-SIGN, I blocked the receptor with mannan or an antibody against DC-SIGN. There was no statistical difference between iDCs infected with JUNVpp and those that were blocked with either mannan or a blocking antibody prior to and during infection (Fig. 2.4B). The presence of DC-SIGN on iDCs was confirmed using immunofluorescence microscopy (Fig 2.5). The absence of L-SIGN was also confirmed using microscopy (data not shown). This data suggests that dendritic cells are not infected by Junín.

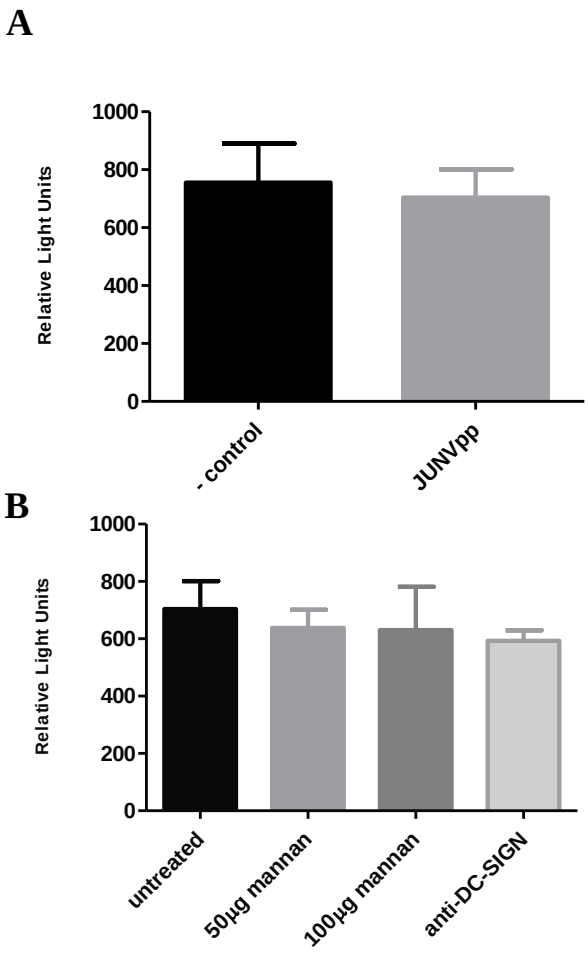

Fig. 2.4 JUNVpp does not transduce iDCs and pretreatment with mannan or antibodies has no effect on transduction. Immature dendritic cells were pretreated with mannose, mannan or anti-DC-SIGN for 1 h. The cells were transduced with JUNVpp in the presence of the blocking agent for 3-4 h. The cells were lysed 48 h post-transduction and luciferase levels were measured as relative light units. All data are the results of a minimum of 3 independent experiments.

JUNVpp *trans*-infects DCs.

Junín has been shown to infect DCs (46) but our data suggest that it is not productively infecting this cell type. Since previous immunofluorescence studies with other viruses showed DCs were *trans*-infected (37, 43, 68), I next transduced iDCs with JUNVpp and checked for the presence of the viral GPC using immunofluorescence (Fig. 2.5). iDCs were transduced for either 4 or 24 h. After 4 h unbound virus was removed by washing with PBS. The cells were stained for expression of the receptors TfR1 and DC-SIGN, an L-SIGN control, as well as the early endosomal compartment, EEA1, and the lysosomal compartment, LAMP-1. Negative controls show the absence of L-SIGN expression but the presence of all other tested receptors and compartments (Fig. 2.5). At 4 hpi, JUNVpp was seen colocalizing with both TfR1 and DC-SIGN receptors and with the EEA1 compartment (Fig. 2.5). At 24 hpi, JUNVpp was still present and was colocalizing with TfR1, DC-SIGN, and EEA1 (Fig. 2.6). However, the virus was not seen within the LAMP-1 compartment at either time point (Fig. 2.5 & 2.6). The virus needs to associate with the late endosome/lysome in order to undergo fusion. JUNVpps continued association with receptors and early endosomal compartments without colocalization with LAMP-1 suggests that the virus *trans*-infects iDCs.

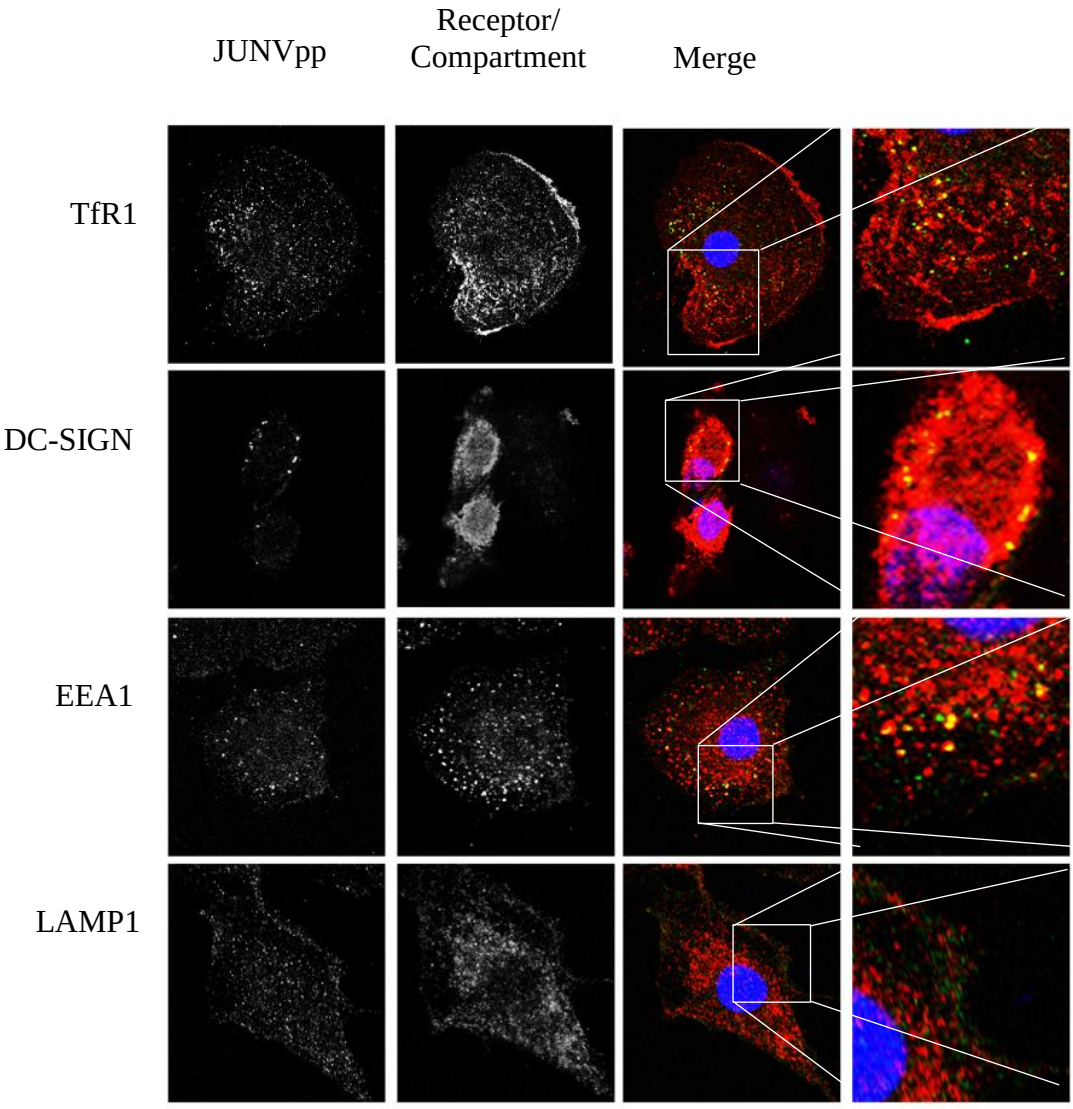

Fig.2.5 JUNVpp colocalizes with DC-SIGN, TfR1 and the early endosomal compartment EEA1 in iDCs by 4 hpi. iDCs were seeded on glass cover-chamber slides and transduced with JUNVpp for 4 h (unbound virus was removed after 3 h). Cells were pre-treated with transferrin-Alexa-fluor 568 for 15 minutes prior to fixation. Cells were immunostained with primary antibodies against DC-SIGN, EEA1 or the lysosomal compartment LAMP-1 as well as Junín GPC. Junín is colored green and tested receptors and compartments are red. Images were visualized on a Leica TCS SP5 Confocal microscope using a 63x oil objective.

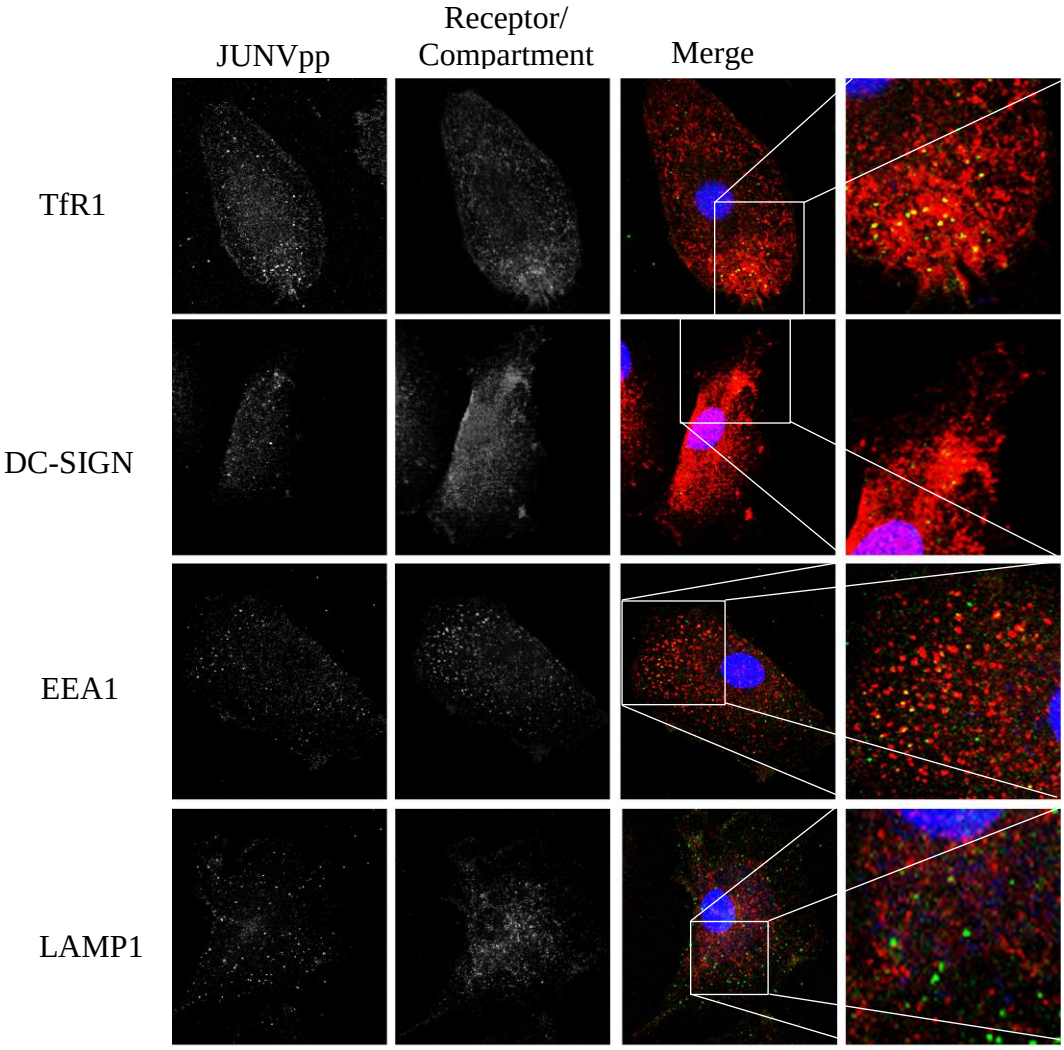

Fig.2.6 JUNVpp colocalizes with DC-SIGN, TfR1 and the early endosomal compartment EEA1 in iDCs by 24 hpi. iDCs were seeded on glass cover-chamber slides and transduced with JUNVpp for 24 h (unbound virus was removed after 3 h). Cells were pre-treated with transferrin-Alexa-fluor 568 for 15 minutes prior to fixation. Cells were immunostained with primary antibodies against DC-SIGN, EEA1 or the lysosomal compartment LAMP-1 as well as Junín GPC. Junín is colored green and tested receptors and compartments are red. Images were visualized on a Leica TCS SP5 Confocal microscope using a 63x oil objective.

Transmission Assays

Some viruses can *trans*-infect dendritic cells in order to transmit the virus to a susceptible cell type. Since our results suggest that iDCs are not productively infected (Fig. 2.4) even though the virus is seen associating with an early endosomal compartment (Fig. 2.5 & 2.6) I attempted to transmit virus from *trans*-infected iDCs to susceptible cells. I transduced iDCs and overlaid them onto cells of varying susceptibility to test if Junín virus could be transmitted. DCs did not transmit JUNVpp to NIH 3T3 or Vero cells. However, JUNVpp was transmitted to primary liver sinusoidal cells, HHSECs (Fig. 2.7). This particular cell type in *vivo* is a target of Junín virus infection.

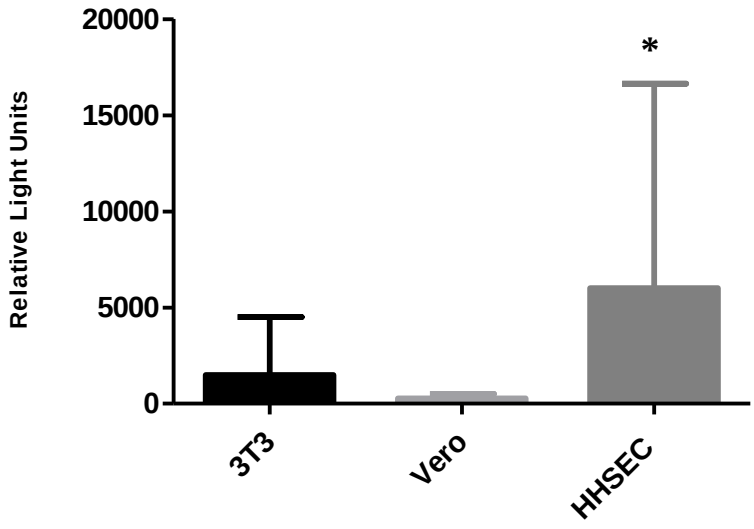

Fig. 2.7 Dendritic cells can transmit JUNVpp to susceptible cell types. Immature dendritic cells were transduced with JUNVpp for 3-4 h. The cells were washed 3 times with PBS then seeded onto monolayers of NIH 3T3, Vero or HHSEC cells. The cell monolayer was washed the following day to remove unbound DCs. The cells were lysed and luciferase activity was measured 48 h later. All data are the results of a minimum of 3 independent experiments. * $p < 0.05$.

Junín Arenavirus Pathogenesis Model

The currently accepted receptor for Junín Arenavirus is TfR1. Infection can occur through inhalation of viral particles. Since TfR1 is located basolaterally in the airway epithelium and unavailable to inhaled virus, I propose that dendritic cells which can periodically sample the airway epithelium can bind and internalize the virus through either its DC-SIGN or TfR1 receptors. The DC can then enter a draining lymph duct and carry the virus to a lymph node where the virus is then handed off to susceptible cells within the node since nodes are a target organ of the virus (Fig. 2.8).

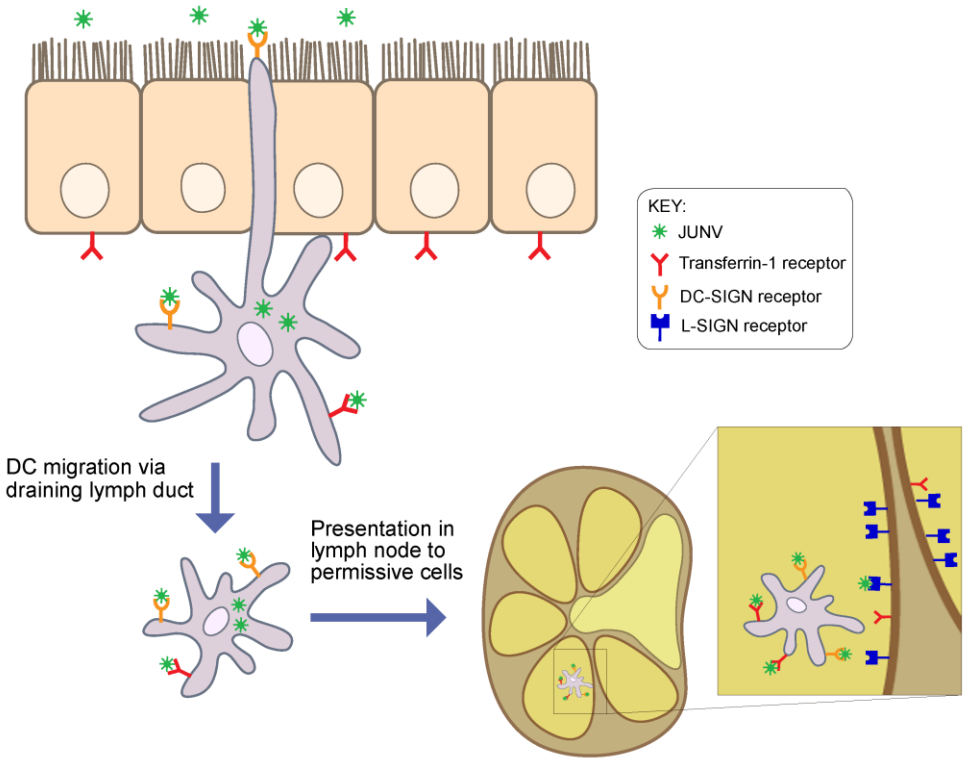

Figure 2.8: **Proposed Junín Pathogenesis Model.** Junín Arenavirus uses the endogenous transferrin receptor of its human host to infect target cells and organs. It is believed that most infections arise from inhaling infected rodent excreta. However, the transferrin receptor is located basolaterally in the airway epithelium, which suggests that the virus may utilize additional receptor(s) to initiate infection. We propose that sub-epithelial dendritic cells, which are known to sample airway epithelium, bind JUNV via DC-SIGN, a C-type lectin used by many viruses, including HIV. Once JUNV binds DC-SIGN, the virus is internalized but does not set up a productive infection. Instead, the virus is transported to the lymph nodes and liver where the virus is then transmitted to receptors.

Discussion

Junín Arenavirus has been isolated from the liver, lymph nodes and the spleen and these organs are considered targets of viral infection. However, viral infection is generally due to inhalation of viral particles. Human TfR1, a previously identified Junín receptor, is located basolaterally in the lungs and is not available to the inhaled virus particles. I am proposing that sub-epithelial dendritic cells become *trans*-infected with Junín while sampling the airway epithelium. The infected dendritic cell will enter a draining lymphatic duct and travel to a nearby lymph node where the virus is disseminated.

Members of the arenavirus family have been shown to interact with the C-type lectin DC-SIGN found on monocyte derived dendritic cells (DCs) and it has been suggested that DCs may disseminate the virus throughout the host (30, 46). To analyze the interaction between Junín and iDCs, I generated pseudoparticles expressing the Junín glycoprotein on an MLV background. Initial experiments with 3T3 cells transiently expressing DC-SIGN, L-SIGN and TfR1 were inconclusive. It has been shown that the internalization pathway of DC-SIGN and L-SIGN is dependent on the cell type (45). My results may have been affected by the ability of the transfected receptors to function in 3T3 cells so I extended my focus to include primary human immature dendritic cells that naturally express DC-SIGN.

The Junín pseudoparticles (JUNVpp) were next used to transduce immature monocyte derived DCs. I demonstrated that JUNVpp does not transduce iDCs, though prior research suggests that DCs are infected by Junín virus (30) (Fig. 2.4). Further investigation demonstrated that JUNVpp associated with both TfR1 and DC-SIGN on iDCs and was targeted to non-lysosomal compartments (Fig. 2.5 & 2.6). However, the pseudoparticles did not progress to late endosomal/lysosomal compartments. Junín fuses at pH<5.5 and the pH of early endosomes

ranges from 6.0-6.8 suggesting that Junín does not undergo fusion in this compartment and instead must progress to a late endocytic compartment (11, 52).

Different viruses, such as HCV, can *trans*-infect dendritic cells by an as yet unknown mechanism(s) to be delivered to a permissive cell type essentially using DCs as Trojan Horses (3, 45, 69). Experiments using antibodies directed to the neck region of DC-SIGN instead of the carbohydrate recognition domain (CRD) have demonstrated that antigen was routed differently within the DC and tended to remain associated with EEA1 compartments instead of routing to the lysosome (65). I believe that Junín Arenavirus is using this mechanism of binding to the neck region of DC-SIGN and instead of moving to the lysosome the virus is alternatively recycling back to the surface of the DC. DC-SIGN seems to function more as a recycling receptor that internalizes the virus then recycles it back to the cell surface. Internalized Junín could then be transmitted to permissive cells in the lymph nodes and the infection would progress to the liver and spleen.

I further show that the endocytosis motif of L-SIGN is important in promoting Junín pseudovirus entry. Whereas mutation of the functional LL endocytosis motif of DC-SIGN showed no significant difference in virus uptake in 3T3 cells, the equivalent mutation in L-SIGN resulted in a significant reduction in virus uptake (Fig. 2.3B). This reduction in uptake was observed despite the increased levels of L-SIGN expression on the cell surface (Fig. 2.3A), suggesting the effective reduction of virus internalization may actually be an under-representation of the actual situation in cells, once cell-surface expression levels are normalized. Overall, I consider that both DC-SIGN and L-SIGN are likely to act as binding factors for the virus, with L-SIGN also having the capacity to act as an alternate Junín virus receptor.

These results suggest that C-type lectins can significantly enhance virus entry and infection in less susceptible cells, which is significant in the study of arenavirus biology VHF viruses, including Junín virus, infect monocytes, macrophages and dendritic cells (DCs) during early stages of the disease (30) and a role for DC or L-SIGN is likely *in vivo*. Recent studies show the ability of LASV and LCMV to enter cells enhanced by DC-SIGN. This finding, together with my present results, demonstrates that members of both New World and Old World arenavirus group can increase their infectivity in the presence of these lectins (62, 63). To date, the actual cell types that utilize a C-type lectin-mediated mechanism for Junín virus entry in the host are not known. DC-SIGN is widely expressed in immature monocyte-derived DCs, and DCs are considered to be infected during VHF (30); however there are other lectins expressed on DCs besides DC-SIGN (21, 26). L-SIGN is expressed in certain types of endothelial cells (5), which are key target cells in Junín virus-mediated hemorrhagic disease (31, 39). In both cases a C-type lectin-mediated entry mechanism may have important implications for Junín virus pathogenesis and the development of VHF.

My study showing an enhancement of the infection in DC-SIGN or L-SIGN positive cells demonstrates the importance of researching JUNV GPC to determine if changes in this binding have an impact on virus infectivity, tropism and pathogenicity. My results suggest that DCs capture Junín arenavirus and disseminate the virus to target organs where productive infection is initiated. I propose that sub-epithelial DCs sampling the bronchial lumen, bind and endocytose Junín arenavirus and subsequently disseminate the virus to target organs as a model of pathogenesis

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Chapter 3

Utilization of LSECtin for entry and infection of host cells by the New World Arenavirus, Junín virus

Introduction

Arenaviridae are RNA viruses which encode a bi-segmented ambisense genome. The L segment encodes genes for the viral polymerase and a zinc-binding protein. The S segment has genes for the viral nucleocapsid and the precursor glycoprotein. The glycoprotein is cleaved into three segments, the stable signal peptide (SSP), GP-1 (receptor binding) and GP-2 (membrane fusion), all of which form the viral glycoprotein complex (GPC). The viral envelope is studded with tripartite GPCs that are involved in viral binding to a receptor and subsequent fusion with a lysosomal membrane (2, 4, 35, 36).

Arenaviruses can be grouped into the Old World and New World serocomplexes with the New World being broken down further into Clades A, B, and C. There are currently 28 identified arenaviruses (6). Some members of both serocomplexes can be transmitted from their natural rodent host to humans resulting in disease that can be severe and occasionally fatal. Pathogenic representative members of the Old World viruses, Lassa and LCMV, utilize the α-dystroglycan receptor. Five of the New World Clade B arenaviruses , namely Junín (JUNV), Machupo (MACV), Sabia (SABV), Chapare (CHPV), and Guanarito (GTOV) utilize the transferrin 1 receptor(TfR1) and cause hemorrhagic fever in humans (27).

Infection generally occurs through inhalation of infected rodent excreta but the exact pathogenesis of disease is not well understood. Examination of tissues from fatal human cases or non-human primates indicate that innate immune system cells (monocytes, macrophages and dendritic cells) and organs such as the liver, lymph-nodes and spleen can be infected with arenaviruses (10, 13, 17, 19) showing that pathogenic arenaviruses, such as Junín, have broad tissue tropism.

However, receptor usage is species specific. Junín utilizes the TfR1 receptor of its natural rodent host, *Calomys musculinus* but not that of *Mus musculinus* (28). Conversely, Junín and Candid1, the vaccine strain of Junín, have been shown to productively infect mouse cells indicating the virus is using an unknown pathway/receptor to initiate infection (7, 11, 14). Previous work from our lab showed enhanced transduction of JUNVpp in cells that express either DC-SIGN or L-SIGN along with TfR1 suggesting roles as attachment factors or recycling receptors (Chapter 1).

Alternate receptors or attachment factors include the C-type lectins, which were originally demonstrated to mediate entry for HIV-1 (3, 8, 12). It is known that viruses in various families use DC-SIGN (CD209) and/or the DC-SIGN homologue, DC-SIGNR also known as L-SIGN (CD209L), for entry and dissemination (5, 22). Recently, C-type lectins were shown to play a role in the infection of Lassa virus and LCMV, both members of the Old World arenavirus group (31, 33).

LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin) has also been shown to interact with ebola, SARS, hepatitis C, Lassa and LCMV arenaviruses (15, 20, 32, 34). LSECtin is found on liver cells, lymph nodes and has been reported to be found on populations of macrophages and dendritic cells and is involved in antigen uptake and internalization (9).

Unlike DC-SIGN and L-SIGN, LSECtin does not bind to high mannose residues (9, 26) and does not appear to release its' ligand upon exposure to the low pH of late endosomes and lysosomes under in vitro conditions (16). LSECtin binds to the disaccharide GlcNAcβ1-2Man with high affinity and to fucose α1-2 (26).

Arenaviruses have also been shown to interact with DC-SIGN, L-SIGN, and LSECtin to enter and infect cells independently of the α-dystroglycan receptor (32, 34). In the context of liver cells, a target of Junín infection, DC-SIGN and L-SIGN function as attachment factors for HCV

infection. To determine the role of all three lectins on Junín infection, I transduced primary human liver sinusoidal cells (HHSECs) with Junín pseudovirus (JUNVpp) and show that the cells are productively infected. HHSECs express DC-SIGN, L-SIGN, LSECtin and TfR1. Anti-LSECtin and GlcNAcβ1-2Man inhibited transduction by JUNVpp suggesting that LSECtin is a major receptor for Junín infection.

Materials and Methods

Cell Culture

HEK 293T/17 (ATCC CRL-11268), Vero E6 (ATCC CRL-1586) and NIH 3T3 cells (ATCC CCL 1658) were grown in DMEM supplemented with 10% FBS, penicillin/streptomycin and HEPES (20 µM). (CHO)-TRVb (Dr. Colin Parrish, Cornell University) were grown in HAMS F-12 Modified medium supplemented with 5% FBS and penicillin/streptomycin. Human hepatic sinusoidal endothelial cells (HHSECs) (ScienCell) were grown in complete endothelial cell medium (ScienCell 1001) and cultured on fibronectin (ScienCell 8248) treated flasks. In all cases, cultures were grown at 37^{o}C in 5% $CO_{2.}$

Plasmids

JUNV GPC was synthesized using the sequence of JUNV strain IV4454, GenBank: DQ272266.3 (GeneArt) and subcloned into the pcDNA3.1 expression plasmid. Human LSECtin was purchased from OriGene (SC307722) and sub-cloned into pcDNA 3.1 (Invitrogen). Human TfR1 (Dr. Colin Parrish, Cornell University) and human DC-SIGN and L-SIGN (NIAID AIDS Research and Reference Reagent Program) were also cloned into pcDNA 3.1.

Reagents and antibodies

Mannan and fucose were obtained from Sigma. GlcNAcβ1-2Man (β1-2 *N*-Acetylglucosamine-mannose) was purchased from V-LABS, INC. Anti-JUNV monoclonal antibodies GB03-BE08 was donated by Dr. A. Sanchez (Centers for Disease Control, Atlanta GA, USA). Polyclonal anti-DC-SIGN (ab97526) anti-L-SIGN (ab58603), anti-Lamp-1 (ab24170) and anti-EEA1 (ab2900) were obtained from Abcam. Monoclonal anti-DC-SIGN (MAB161) and anti-L-SIGN (MAB162) used for blocking were purchased from R&D Systems. Polyclonal LSECtin (sc-70177) was purchased from Santa Cruz. Alexa Fluor secondary antibodies, Alexa Fluor 568-conjugated transferrin, and Prolong Gold Antifade with Dapi were obtained from Molecular Probes.

Production of JUNV pseudotyped retrovirus

HEK 293T cells were co-transfected with equal amounts of an MLV-based transfer vector encoding luciferase, an MLV Gag-Pol packaging construct, and an envelope glycoprotein expressing vector, pcDNA3.1-JUNV GPC, using Turbofect (Fermentas), as recommended by the manufacturer. Cells were incubated at 37°C for 48 h after transfection. Upon harvesting, the supernatants were filtered through 0.45-μm pore sized membranes (Sarstedt). JUNV pseudovirus (JUNVpp) was used immediately or concentrated using sterile polyethylene glycol (PEG 8000) and stored at –80^{o}C for future use.

C-type lectin expression

3T3 or TRVb cells in 24 well plates were transiently transfected with 500 ng of TfR1, DC-SIGN, L-SIGN, LSECtin and/or empty pcDNA3.1 vector singly or in combination using Turbofect or Lipofectamine 2000 (Invitrogen) respectively. To allow for sufficient expression, cells were

incubated for 18-24 h post transfection then split using Trypsin EDTA into 96 well plates and incubated for another 18-24 h. Cells were transduced for 2-3 h with JUNVpp, virus was removed and cells were re-fed with complete medium. Cells were lysed with Lysis Buffer (Promega) 48 h after transduction and luciferase activity was measured with the resulting supernatant using a Luciferase Assay Kit (Promega) and a Glomax 20/20 luminometer (Promega).

Transduction of human hepatic sinusoidal endothelial cells

Human hepatic sinusoidal endothelial cells (HHSECs) were seeded onto fibronectin coated 96 well plates. Cells were transduced with JUNVpp for a maximum of 4 h. The cells were washed with sterile PBS to remove unbound virus and allowed to incubate for a total of 48 h before luciferase activity was measured using a Luciferase Assay Kit (Promega) and light emission measured by using a Glomax 20/20 luminometer (Promega).

Carbohydrate and antibody blocking assays of primary cell receptors

HHSEC cells seeded onto fibronectin coated 96 well plates were pretreated with 50 or 100 μg/mL of fucose, mannan or GlcNAcβ1-2Man or 5 or 10 μg/mL anti-DC-SIGN, L-SIGN, LSECtin or TfR1 for a minimum of 1 h. After pre-treatment, JUNV pseudovirus was added in the presence of fresh blocking agent and incubated for 2-4 h. Cells were washed with PBS to remove unbound virus and then re-fed with additional blocking agent. Cells were harvested 48 h later and luciferase activity was measured.

Colocalization assay of JUNVpp with receptors and cellular compartments

HHSECs seeded on fibronectin coated chamber slides were transduced with JUNV pseudovirus. Cells were fixed with 4% paraformaldehyde at 4 and 24 hpi. Fifteen minutes prior to fixation, select wells were treated with Alexa Fluor 568-conjugated transferrin at 37^{o}C. Cells were treated

with primary antibodies against JUNV-GPC, LAMP-1, EEA-1, DC-SIGN, L-SIGN and LSECtin. Alexa-fluor 488 was used to visualize JUNV-GPC and Alexa-fluor 568 was used to visualize all tested receptors and cellular compartments. Fixed slides were imaged as z-stacks with a Leica TCS SP5 Confocal Microscope.

Statistical methods

All experiments were conducted at a minimum of three independent trials. Graph Pad Prism 5 was used to produce all graphs and calculate statistics. All significance levels were calculated at $p < 0.05$. Tukey Post tests were run to compare between groups.

Results

JUNVpp can successfully transduce primary HHSEC cells.

Since neither 3T3 nor TRVb cells naturally express DC-SIGN, L-SIGN or LSECtin, I utilized a primary human liver sinusoidal cell line (HHSEC). The liver is a target organ that has a high viral load in a natural Junín infection and sinusoidal liver cells have been reported to express all three C-type lectins. HHSEC cells were transduced with JUNVpp for 2-3 hours. The pseudovirus virus inoculum was removed and luciferase activity was measured 48 h later. Transduced HHSEC cells were highly transducible when compared to the uninfected control and were graphed on a log scale (Fig. 3.1).

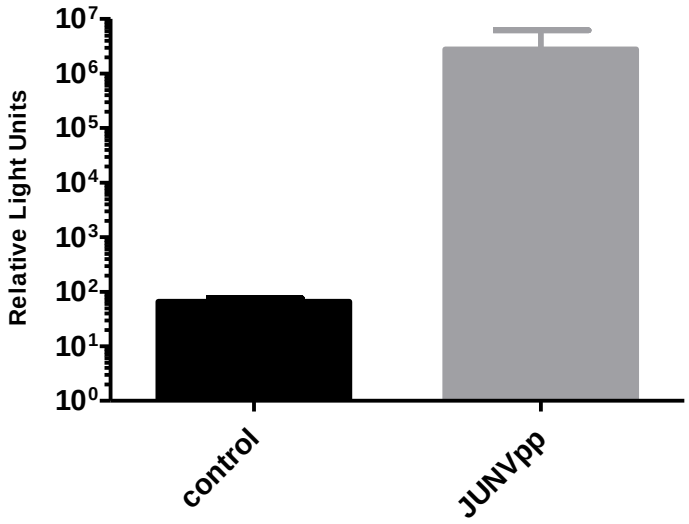


Fig. 3.1 HHSEC cells are transducible by JUNVpp. HHSEC cells were transduced with JUNVpp for 2-3 h, washed and re-fed with complete medium. Cells were lysed 48 h post transduction and luciferase activity was measured. Untreated cells were included as a control to measure infectivity. The graph was constructed as a log scale. All data are the results of a minimum of 3 independent experiments.

JUNVpp colocalizes with HHSEC C-type lectins and endocytosis compartments. Since HHSECs are easily transducible, I performed immunofluorescent colocalization experiments using confocal microscopy in an effort to determine which receptor(s) are utilized by Junín in HHSEC cells. Once Junín binds to a receptor, it is endocytosed into the interior of the cell (30). I stained for the early endosomal compartment (EEA1) and a late endosomal/lysosomal compartment (LAMP-1) to visualize if the virus was being internalized or if it remained bound to a receptor on the cell surface. Junín virus must be endocytosed to a late endosomal compartment in order for pH-dependent membrane fusion to occur. HHSEC cells were transduced with virus and the infection was allowed to proceed for 4 h. Fifteen minutes prior to harvesting, transferrin bound Alexa-fluor 568 was added to TfR1 assigned wells. JUNVpp was seen colocalizing with the receptors TfR1, DC-SIGN, L-SIGN and LSECtin (Fig. 3.2) and was also seen colocalizing in EEA1 and LAMP-1 cellular compartments (Fig. 3.3).

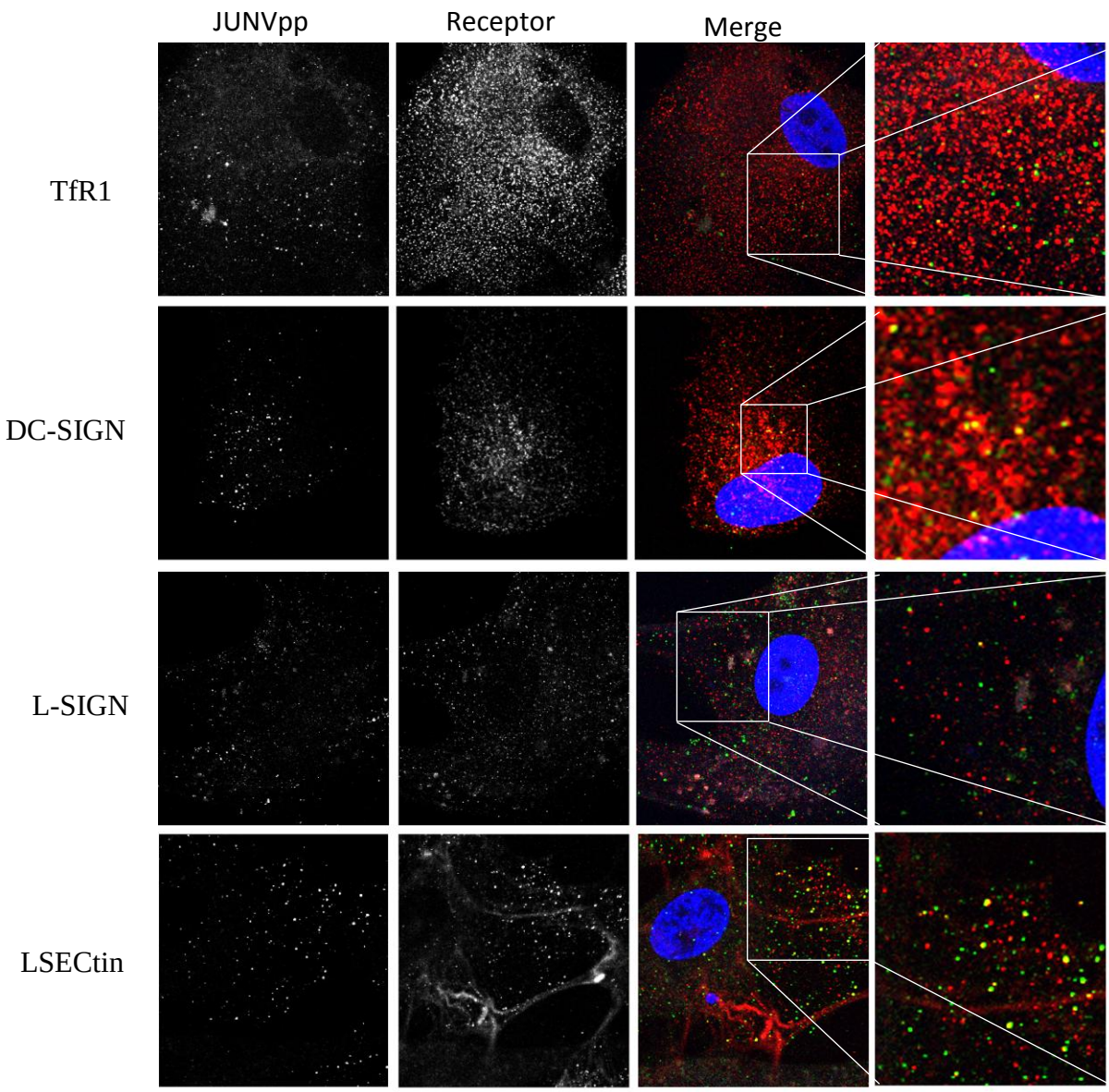


Fig. 3.2 JUNVpp colocalizes with cellular receptors TfR1, DC-SIGN, L-SIGN and LSECtin. Junvpp was used to transduce HHSEC cells for 4 h. Fifteen minutes prior to fixation, transferrin-568 was added and used to identify TfR1. Cellular receptors are colored red and JUNVpp is colored green. Images were analyzed using a Leica confocal microscope 63x oil objective.

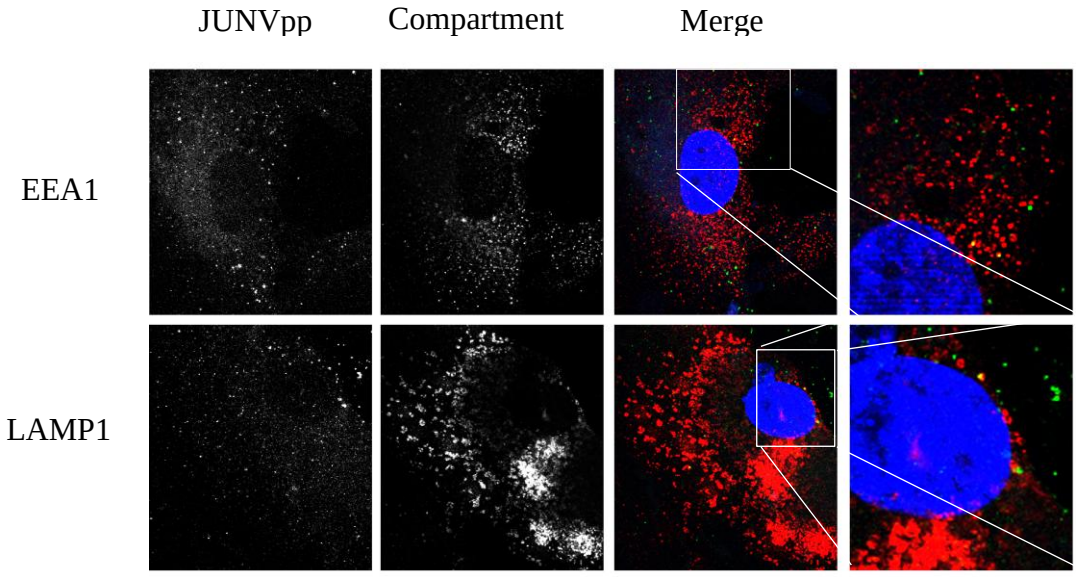


Fig. 3.3 **JUNVpp colocalizes with endocytic compartment markers EEA1 and LAMP1.** Junvpp was used to transduce HHSEC cells for 4 h. Cells were fixed and stained using antibodies specific to each compartment. Cellular compartments are colored red and JUNVpp is colored green. Images were analyzed using a Leica confocal microscope 63x objective.

Pretreatment with GlcNAcβ1-2Man and anti-LSECtin significantly decreases JUNVpp transduction of HHSEC cells.

Since JUNVpp can colocalize and be endocytosed by TfR1, DC-SIGN, L-SIGN and LSECtin as receptors I attempted to individually block these receptors to determine the effect each receptor has on entry. I used mannan, fucose and GlcNAcβ1-2Man as well as antibodies against TfR1, DC-SIGN, L-SIGN and LSECtin to block viral attachment. DC-SIGN binds high mannose and fucose residues and mannan has been used to inhibit binding to the receptor (25). L-SIGN also binds mannose residues (25). LSECtin binds N-Acetylglucosamine (GlcNAc), mannose, fucose and GlcNAcβ1-2Man (21, 26). The TfR1 receptor binds transferrin; however, crystallography has shown that Machupo Arenavirus binds to a different region on TfR1 than does transferrin (1). Transferrin and JUNVpp can both bind to the receptor at the same time as shown by colocalization experiments (Fig. 3.2). HHSEC cells were blocked with inhibitor for a minimum of 1 h prior to addition of virus and cells were maintained in blocking agent throughout the experiment. There was a dose dependent reduction in luciferase activity for all carbohydrates tested but only 100μg GlcNAcβ1-2Man was significant (Fig. 3.4A). All blocking antibodies at all concentrations tested showed significant reduction in the ability of JUNVpp to transduce HHSEC cells but anti-LSECtin is the most effective at both concentrations tested (Fig. 3.4B). However, antibody affinities are not known and may play a role in the ability to block viral binding and entry,

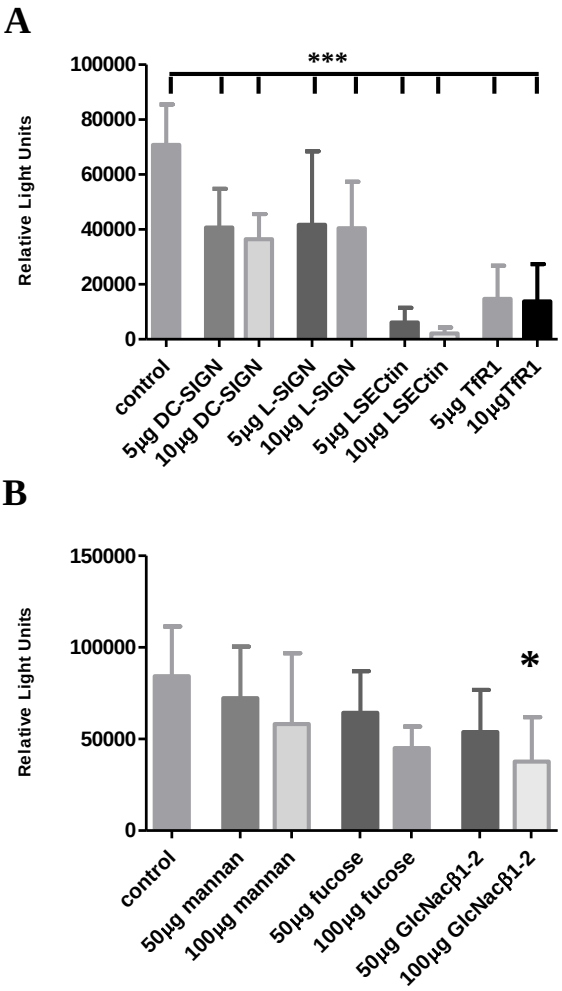
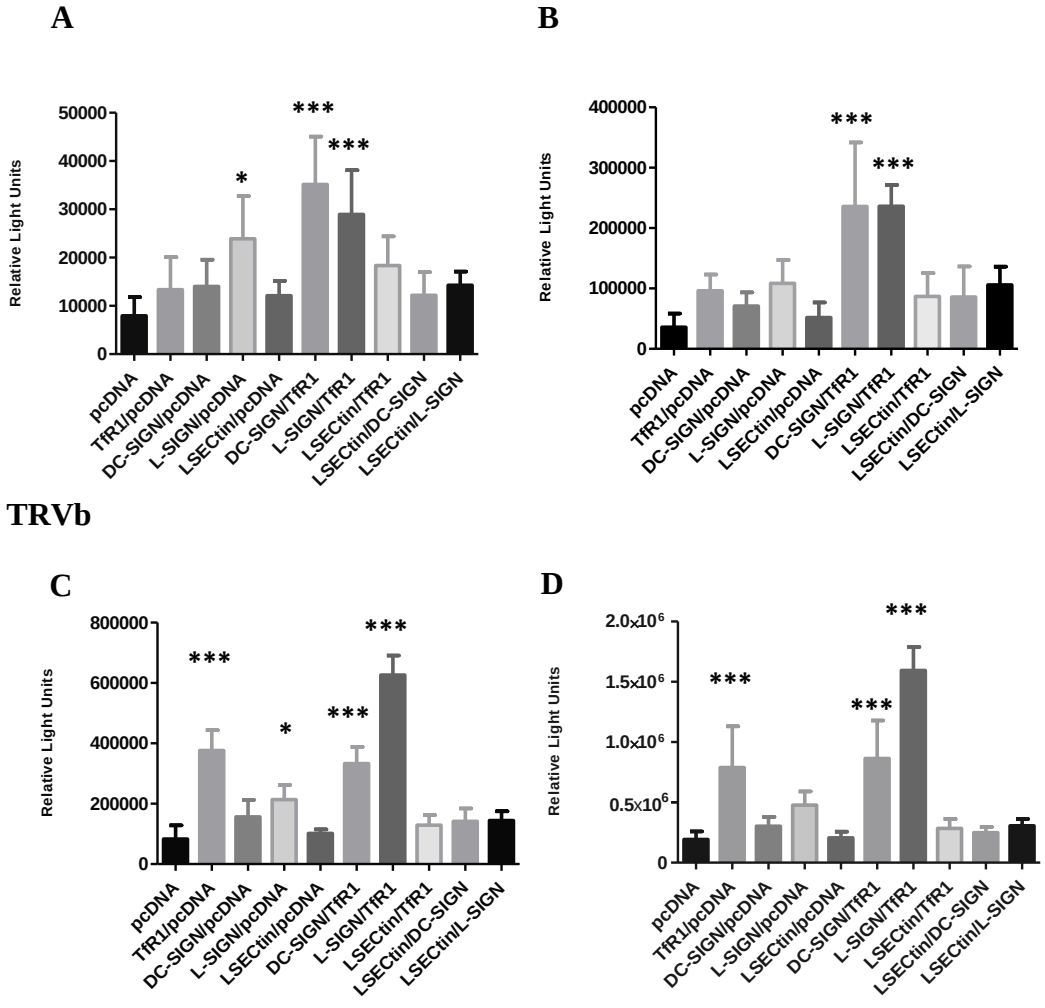


Fig. 3.4 Blocking the LSECtin receptor significantly reduces transduction of HHSEC cells. HHSEC cells were pretreated for 1 h with 2 different concentrations of receptor specific antibodies (A) or carbohydrates (B). The cells were transduced with JUNVpp in the presence of blocking agent, washed then re-fed with block. Luciferase activity was measured 48 h post transduction. All data are the results of a minimum of 3 independent experiments and was analyzed using One-way ANOVA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

DC-SIGN and L-SIGN enhance transduction of semi-permissive cells.

To further elucidate our understanding of the role of LSECtin in Junín virus infection, NIH 3T3 and TRVb cells were used to test interactions between JUNVpp and the C-type lectin. The relatively non-susceptible 3T3 cells can be infected with Junín through an as yet undetermined route since the virus does not utilize *Mus musculus* TfR1 (7, 11, 29). To control for the possibility that JUNVpp was utilizing the host cell transferrin receptor I repeated experiments with TRVb cells which do not express an endogenous TfR1 (24). Additionally, I transduced both cell types with low and high titers of virus to see if that played a role in receptor usage. Expression of TfR1 and L-SIGN showed a significant increase in luciferase activity in TRVb cells (Fig. 3.5 C & D). Both TRVb and 3T3 cells also showed a significant increase ($p < 0.005$) in luciferase activity in cells that were doubly transfected with either TfR1 and DC-SIGN or TfR1 and L-SIGN when compared to cells not transfected with a receptor (Fig. 3.5). 3T3 cells showed a significant difference between cells doubly transfected with pcDNA3.1/TfR1 compared to those transfected with TfR1/DC-SIGN or TfR1/L-SIGN while TRVb cells only showed a significant increase in TfR1/L-SIGN cells (Fig. 3.5). The transfection of pcDNA3.1-LSECtin had little effect in JUNVpp's ability to transduce either cell type (Fig. 3.5). Interestingly, there were differences in results between 3T3 and TRVb cells indicating that transfected receptors function differently between cell types (Fig. 3.5). Based on this data, I believe that the plasmid of LSECtin is functioning as a dominant negative in the context of NIH 3T3 and TRVb cells. This is not unusual since receptors transfected into non-native cell types may not function as they would in a natural host cell as has already been shown for DC-SIGN and L-SIGN (23).

NIH 3T3



TRVb

Fig. 3.5 DC-SIGN and L-SIGN enhance transduction of semi-permissive cells. Combinations of receptors and empty pcDNA3.1 vector were transfected into NIH 3T3 cells (Figs. A & B) or TRVbs. (Figs. C & D). JUNVpp was used to transduce the cells at either low (Figs. A & C) or high titer (Figs. B & D). Luciferase activity was measured 48 h post transduction. All data is the results of 3 independent experiments and was analyzed using One-way ANOVA. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

In this paper, I have identified LSECtin as being a receptor for JUNVpp infection. LSECtin is found on liver sinusoidal endothelial cells as well as in lymph nodes. Junín Arenavirus has been isolated from the liver, lymph nodes and the spleen and these organs are considered targets of viral infection.

I opted to use a primary human liver sinusoidal endothelial cell which shares many of the same receptors as lymph nodes and is also a site of Junín infection to avoid artificial conditions and complications that can arise from transfecting a receptor into a cell type. Previous work has established that the C-type lectins DC-SIGN and L-SIGN are internalized differently based on cell type (23). The HHSEC cells naturally express the C-type lectins DC-SIGN, L-SIGN and LSECtin. JUNVpp colocalized with all of these receptors and also with TfR1. Production of luciferase implies that the virus is routed through the endocytic pathway and undergoes fusion in an acidified compartment (Fig. 3.1). JUNVpp was also seen colocalizing with early endosomal (EEA1) and late endosomal/lysosomal compartments (LAMP-1) (Fig. 3.3). I saw significant decreases in infectivity when all receptors were blocked with antibodies but the blocking assays with anti-LSECtin and GlcNAcβ1-2Man significantly inhibited transduction in a manner similar to inhibition of TfR1 indicating that LSECtin is a receptor for Junín Arenavirus (Fig. 3.4).

In previous work, I showed that immature dendritic cells are not productively infected with JUNVpp (Chapter 1). I proposed that sub-epithelial dendritic cells become *trans*-infected with Junín while sampling the airway epithelium. The infected dendritic cell will enter a draining lymphatic duct and travel to a nearby lymph node where the virus is disseminated and infects the lymph node. LSECtin is located within the lymph nodes and could potentially be involved in the infection process within that organ. Once virus is disseminated into the blood stream it could

travel to the liver as part of normal circulation. Sinusoidal endothelial cells located within the lumen of the liver function as a barrier between macromolecules found in the blood and hepatocytes. They also can function as antigen presenting cells and have a major role in clearing macromolecules from the blood (18). Since viruses have subverted other antigen presenting cells it is not unreasonable to think that Junín Arenavirus has subverted HHSEC cells via the LSECtin receptor.

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Chapter 4

Junín Arenavirus GP-1 N-linked Glycosylation Sites are Crucial for Glycoprotein Processing and Function

Introduction

Junín Arenavirus is an RNA virus with a bipartite genome that encodes 4 genes: the matrix protein (Z), the RNA-dependent RNA polymerase, the nucleoprotein (NP), and the glycoprotein (GPC). The glycoprotein is produced from a single chain precursor into a complex consisting of a stable signal peptide (SSP), a GP-1 subunit responsible for receptor binding and the GP-2 subunit that is involved in membrane fusion within an acidified endosome. The SSP is unusual in that it remains associated with the glycoprotein once it is cleaved by SPase within the lumen of the endoplasmic reticulum (20). SSP is critical for trafficking the glycoprotein to the cell membrane and fusion with the host cell membrane within the lumen of an endosome (3, 4).

The GP-1 and GP-2 subunits are cleaved by the cellular protease SKI-1/S1P in the Golgi (31). GP-1 forms a non-covalent bond with GP-2 and the SSP is also non-covalently bound to GP-2. Once precursor GPC is cleaved into the GP-1/GP-2/SSP tripartite complex (GPC), the glycoprotein can trimerize in the secretory pathway and be escorted to the surface (40). The virus is assembled and buds at the membrane surface through interactions with the Z protein (2, 11, 12).

New World arenaviruses utilize the transferrin 1 (TfR1) receptor of their natural rodent host. Additionally, pathogenic New World arenaviruses, such as Junín and Machupo, can utilize human TfR1 as a receptor (13, 19, 39). A crystal structure of Machupo bound to TfR1 has been solved and the key residues involved in TfR1 binding have been identified (1).

However, many viruses, including HIV, HCV and ebola can use multiple receptors to bind and enter host cells via their envelope glycoprotein (14, 21, 33, 43). Viral glycoproteins can be glycosylated at an Asn-Xxx-Ser/Thr motif within the lumen of the E.R. This N-linked

glycosylation has been linked to both protein structure and function, viral binding to host cell receptors and can also play a role in development of neutralizing epitopes (25, 26, 37, 38). Core oligosaccharides are added to the protein and eventually cleaved to a high mannose structure before exiting the ER. Once in the Golgi, the N-linked glycan is further modified by the addition/removal of sugars such as fucose, galactose and sialic acid (26). The modified glycans on the glycoprotein can interact with cellular receptors such as the C-type lectins DC-SIGN, L-SIGN, and LSECtin which can function as entry receptors or attachment factors. Lymphocytic choriomenigitis virus (LCMV) and Lassa virus, both Old World arenaviruses, interact with DC-SIGN and LSECtin potentially through N-linked glycans (41, 42). Mutations of N-linked glycans on Lassa virus GPC identified 6 (out of 11 total) N-linked glycosylation sites as being critical for GPC cleavage (17). Whereas, only 2 (out of 8) LCMV glycans were critical for cleavage but infectivity was impaired when the other glycans were removed (8).

C-type lectins are calcium dependent and have a carbohydrate recognition domain (CRD) that can bind to carbohydrate groups found on glycans on pathogens (10, 18, 32). DC-SIGN and L-SIGN are C-type lectins that can function as attachment factors or receptors in viral binding and entry (6, 7, 16, 21, 24, 36, 44). DC-SIGN binds fucose and mannose while L-SIGN binds to mannose oligosaccharides (23). Glycosidase digestion of Junín suggests that GP-1 has mannose and galactose residues (22).

Junín GP-1 is predicted to have 4 N-linked glycosylation sites and the GP-2 subunit has 3 based on sequence alignment and use of an N-linked glycosylation prediction program, NetNGlyc. In the present study, I mutated the asparagine residues in GP-1 to glutamines to prevent addition of glycans to determine if these sites are important for maintaining structural integrity or involved in viral entry. I generated pseudotyped viral particles containing WT or mutant GPC to evaluate

the role of N-linked glycosylation on viral entry. Our results suggest that sites N95 and N178 are critical for GPC structure with N166 less so while sites N105 and N166 are involved in entry events.

Materials and Methods

Cells

NIH 3T3 cells (ATCC CCL 1658) and HEK 293T/17 cells (ATCC CRL-11268) were cultured in DMEM (CellGro) supplemented with 10% fetal bovine serum (FBS), 20 μm Hepes and penicillin/streptomycin at 37^{o}C in 5% CO_2.

Mutations

Junín Arenavirus glycoprotein (GPC) strain IV4454, GenBank: DQ272266.3 was synthesized (GeneArt) with a myc-tag at the C-terminus and sub-cloned into pcDNA3.1. Potential glycosylation sites were predicted using NetNGlyc at positions N95, N105, N166 and N178 (http://www.cbs.dtu.dk/services/NetNGlyc/). QuikChange (Agilent) was used to design single point mutation primers that removed the potential glycosylation site using PfuUltra (Agilent) polymerase. All sites were mutated from an asparagine to a glutamine to maintain structure and polarity. Mutations were confirmed by sequencing.

Biotinylation, deglycosylation and Western Blotting of cell surface proteins

HEK 293T cells were transfected with WT and mutant JUNV GPCs. At 24 h post-transfection, the cells were washed with PBS and incubated with EZ-link sulfo-NHS-SS biotin (ThermoScientific). The cells were washed twice with glycine then lysed with 1%NP40/TBS containing Complete Protease Inhibitor (Roche). The cell lysate was centrifuged at 14000 rpm

for 20 min at 4°C. The cleared lysate was precipitated using Pierce Streptavidin Agarose Resin (ThermoScientific) overnight at 4°C with continuous agitation. An aliquot of unbound lysate was reserved to represent cellular lysate. The resin was washed, dried and resuspended in reducing sample buffer. Proteins were separated on a 10% polyacrylamide gel, blotted onto nitrocellulose and visualized with either primary monoclonal or polyclonal anti-myc antibody and a horseradish peroxidase conjugated secondary. Myc-tagged proteins were detected using Pierce ECL Substrate (ThermoScientific) and a Fuji Las 3000 image reader. Precipitated WT GPC was treated with the glycosidases Endo H or PNGaseF (Fermentas) overnight and then separated by SDS-PAGE and immunoblotted as described above.

Pseudovirus Production

HEK 293T cells were transfected using Turbofect (Fermentas) and nanogram equivalent amounts of WT or mutant Junin GPC, the MLV packaging construct Gag-Pol, and a luciferase reporter construct. The glycoprotein from VSV (strain Indiana) was included as a control. Viral containing supernatant was harvested 48 h post-transfection and filtered through a 0.45μm filter (Sarstedt) to remove cellular debris. The cleared Junín pseudoviral (JUNVpp) containing supernatant was used immediately for transductions.

Additional pseudovirus was concentrated using PEG 8000 (Sigma) suspended in TBS. The pseudovirus/PEG solution was rocked overnight at 4°C and centrifuged at 4000 rpm for 30 min at 4°C. The solution was removed without disturbing the pellet and the pellet was washed with PBS then resuspended in PBS. The concentrated virus was run on a Western Blot and visualized as described above.

Transduction Assay

NIH 3T3 cells were transiently transfected with pcDNA3.1 expression vectors encoding the human cellular receptors transferrin 1 (TfR1), DC-SIGN and L-SIGN. Approximately 18 h after plating, 500ng of each receptor in combination with another receptor or empty pcDNA3.1 expression vector (Invitrogen) were transfected using Turbofect (Fermentas) per manufacturer's instructions. Cells were transduced 24 h post-transfection with pseudovirus for approximately 4 h, washed with PBS and refed with complete DMEM. The cells were washed and lysed 48 h post-transduction. The cellular lysate was analyzed using a Luciferase Assay Kit (Promega) and light emission measured by using a Glomax 20/20 luminometer (Promega).

Alignments

Arenavirus glycoprotein sequences Junín (IV4454, DQ272266.3), Junín (MC2, D10072.2), Machupo (Carvallo, NC_005078), Sabia (NC_006317), Guanarito (NC_005077), Chapare (NC_010562), Amapuri (NC_010247), Lassa (Josiah, J04324.1), LCMV (Armstrong, P09991.1) obtained from GenBank were aligned using ClustalW (30).

Molecular Modeling

The reference sequence was adjusted to reflect on the change of each asparagine to a glutamine. The sequence was submitted to CPHmodels (34) and the solved crystal structure of Machupo (PDB, 2WFO) was used as a template (9). I used Pymol (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC) to view the modeled structure.

Results

Comparison of N-linked glycosylation between Arenavirus strains.

For the context of this study, I focused on glycosylation within GP-1 since I was interested in the effects of glycosylation on viral entry (Fig. 4.1). I compared various strains of Junín Arenavirus to our selected strain, IV4454, to determine if there were potential glycosylation sites and if they were conserved across strains. All of the Junín strains analyzed, ranging from the virulent Romero and MC2 strains to the vaccine strain Candid1, had 4 predicted glycosylation sites (N95, N105, N166, N178) on GP-1 except for Romero and Candid1 which lacked glycosylation on N166 site (Fig. 4.2). All strains except IV4454 had a serine at position 107 while IV4454 had a threonine (Fig. 4.2). This amino acid difference did not affect the potential for glycosylation at N105 since N-linked glycosylation is predicted at the peptide sequence Asn-Xxx-Ser/Thr with Xxx being any amino acid except proline. I next compared Junin strain IV4454 with select representatives of both Old and New World arenaviruses. There are varying levels of glycosylation homology across Arenavirus strains with GTOV having 3 predicted sites and SABV, CHPV, and LASV having 6 within the GP-1 subunit (Fig. 4.2). There is a basic conservation of the predicted Junin glycosylation sites (N95, N105, N166, N178) compared to all the arenaviruses I sampled. There is also conservation of predicted glycosylation between viruses that have additional sites. AMAV, SABV, GTOV and CHPV have an additional site at N125-128. Lassa and LCMV have 4 additional sites not shared with Junin. The only GP-1 site that is shared between Junin, Lassa and LCMV is N166. Interestingly, GP-2 predicted glycosylation sites are highly conserved across all samples (Fig. 4.2).

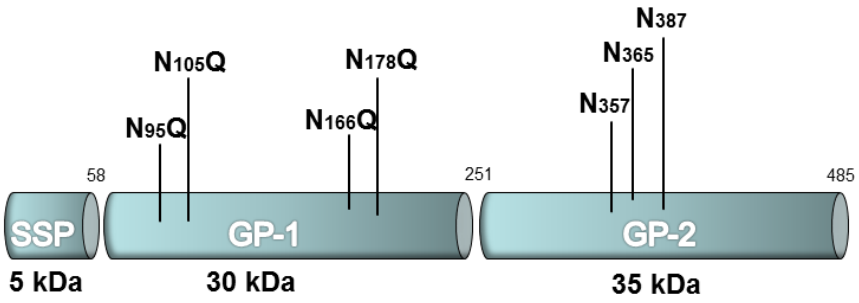

FIG. 4.1 Schematic diagram of Junin virus Glycoprotein. The GPC is produced as a 485aa precursor. The 58aa stable signal peptide (SSP) is cleaved by SPase after the nascent protein is translocated into the lumen of the ER. SSP associates with the C-terminus of GP-2. The complex is folded and escorted out of the ER into the Golgi where GP-1 is cleaved from GP-2 by SKI-1/S1P. GP-1 binds non-covalently to GP-2 and the GPC complex is transported to the cell surface.

Predicted N-glycosylation Sites

Junin Arenavirus Strains	GP-1										GP-2					
IV4454			95	105				166	178				357	365		387
MC2			95					166	178				357	365		387
Romero			95	105					178				357	365		387
XJ13			95	105				166	178				357	365		387
Candid1			95	105					178				357	365		387
Arenavirus Strains																
Junin IV4454			95	105				166	178				357	365		387
Amapari				99			128		174	214	315	352	360			
Machupo		83	95				137	166	178				368	376	393	398
Sabia	69			99			125		171	178	222		360	368		390
Guanarito			88				125		174		314	351	359	376		381
Chapare	69		88	99			125		171	178			356	364		386
Lassa		79		99	109	119			167		224		365	373		395
LCMV		85			114	124			171		232		371			401

Fig. 4.2 Predicted N-linked glycosylation sites for selected Old World and New World arenaviruses. Strains of Junin Arenavirus GPCs were aligned using ClustalW to determine sequence and potential glycosylation homology. The lab strain, Junin IV4454, was then compared to representative Arenaviruses across Old and New World species. Glycosylation sites were determined using NetNGlyc.

Junín GPC is sensitive to treatment with glycosidases.

Myc-tagged WT Junín pcDNA3.1 GPC was transiently transfected into HEK 293T cells using the expression vector pcDNA3.1. Surface proteins were biotinylated and precipitated using strep-avidin agarose resin. GPC was treated overnight with either PNGaseF or EndoH and visualized with immunoblotting. Uncleaved GPC is seen as a faint band at 68 kDa and GP-2 is visible at 35 kDa. GP-1 is not visible since the myc-tag is associated with GP-2. Both GPC and GP-2 are sensitive to digestion by both glycosidases as evidenced by altered migration compared to the untreated control GPC suggesting that the protein is glycosylated (Fig. 4.3).

N-linked-glycosylation site mutants N95Q and N178Q have impaired GPC cleavage.

To investigate if each of the potential glycosylation sites identified by NetNGlyc were involved in viral entry, I mutated each designated asparagine to a glutamine to conserve charge and basic structure since the two amino acids are similar except for an additional methylene group on the glutamine (25). Mutants were designated N95Q, N105Q, N166Q and N178Q. Wild type (WT) and mutant GPCs were transiently transfected into 293T cells. The proteins were biotinylated 24 h post transfection and analyzed by Western Blotting. Uncleaved WT GPC is approximately 68 kDa while all of the mutants are approximately 65 kDa corresponding to a lack of a single glycosylation site (Fig. 4.4A). This suggests that the predicted sites are glycosylated in the WT GP-1. To identify GPC on a Western Blot, I added a myc-tag onto the C-terminus of GPC. When the glycoprotein (GPC) is cleaved into GP-1 and GP-2, the myc-tag is associated with the GP-2 portion of the glycoprotein. Presence of a GP-2 band at 35 kDa indicates that GPC has been cleaved. Mutant N105Q has a cleavage product similar in intensity to WT GPC. N166Q cleavage is impaired and removal of asparagine residues N95 and N178 adversely affects the cleavage of

GPC as the majority of the protein is in an uncleaved state (Fig. 4.4A). The cellular lysate that was reserved from the biotinylation assay contained mainly uncleaved GPCs though faint bands representing cleavage products were visible for WT, N105Q and N166Q (Fig. 4.4B). This result was expected since the glycoprotein had not been fully processed and moved to the plasma membrane. This result also suggests that there is no impairment in protein translation but there is impairment in cleavage and surface expression for mutants N95Q and N178Q.

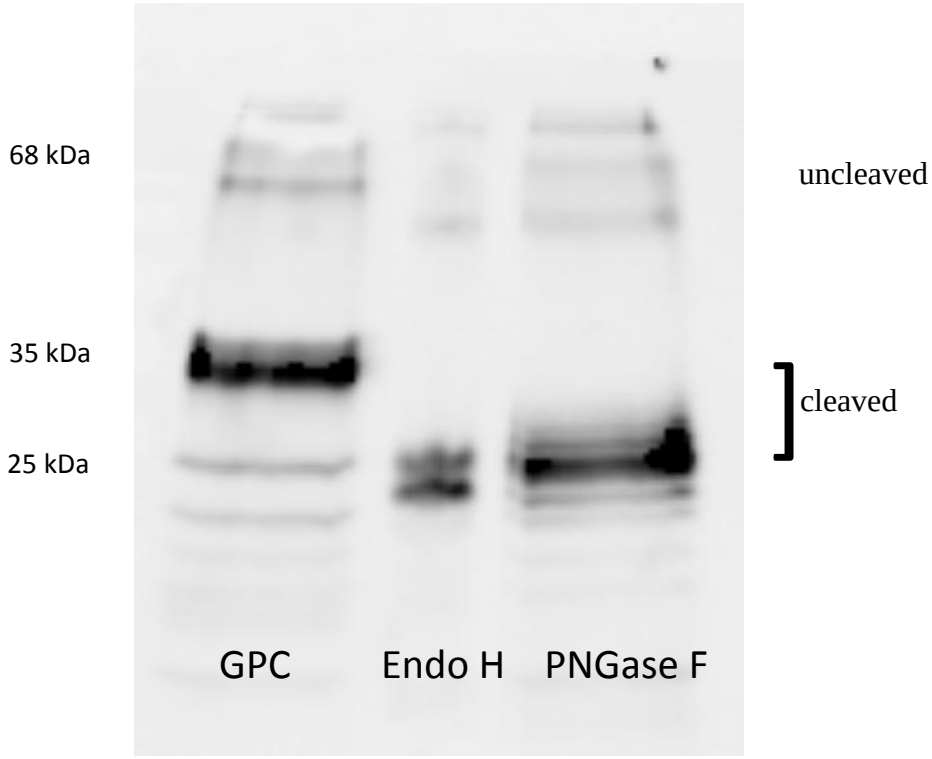

Fig. 4.3 Glycosidase treatment of WT Junín GPC. Biotinylated GPC was treated with EndoH or PNGaseF and separated on a 10% SDS-PAGE gel. Polyclonal anti-myc was used to label the proteins.

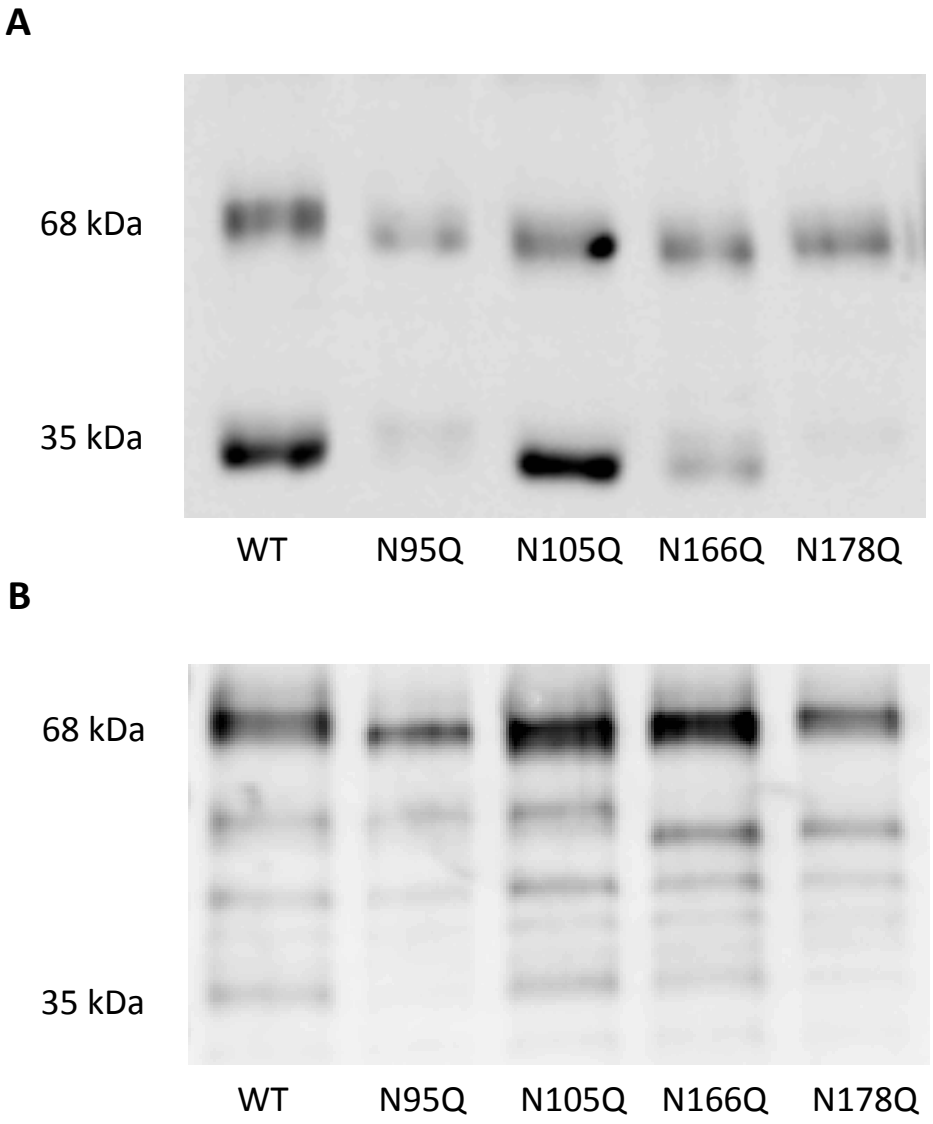

Fig. 4.4 Expression of WT and N-glycosylation deficient mutants. At 24 h post-transfection, 293T cells were biotinylated and the surface proteins were separated from the cellular lysate using strep-avidin agarose beads. Proteins were separated using SDS-PAGE, transferred to nitrocellulose and probed with anti-myc antibody. (A) GPCs expressed on the cell surface. (B) Cellular lysate representing the intracellular fraction of WT and mutant GPCs.

WT and mutants N105Q and N166Q cleaved GPCs are incorporated into pseudoviral particles.

Since there was a defect in cleavage of the mutants, I next tested if cleaved mutant GPC was being incorporated into pseudovirus particles. Virus containing supernatant was concentrated using PEG 8000. VSV-G pseudovirus was included as a control. WT and mutants N105Q and N166Q cleaved GPC were incorporated into the virion as shown by presence of myc-tagged GP-2. There were extremely faint bands for N95Q and N178Q. There were very faint bands at 68kDa for WT and mutant GPCs suggesting that some uncleaved GPC is incorporated into the pseudovirion (Fig. 4.5).

All GPC mutants have impaired infectivity compared to WT.

Known Junín receptors and attachment factors were transfected into a relatively non-permissive cell type. WT and mutant GPC pseudoviruses were used to transduce the cells to determine the effect of N-linked glycosylation on viral entry. All mutants had impaired infectivity as measured by luciferase assay (Fig. 4.6). Mutants N95Q and N178Q showed the most impairment which was expected based on the amount of glycoprotein incorporated into the pseudovirus (Fig.4.5). Based on the data, all of the glycoproteins are important either for involvement in folding and structure or for entry since the loss of a single glycosylation site inhibits viral infectivity.

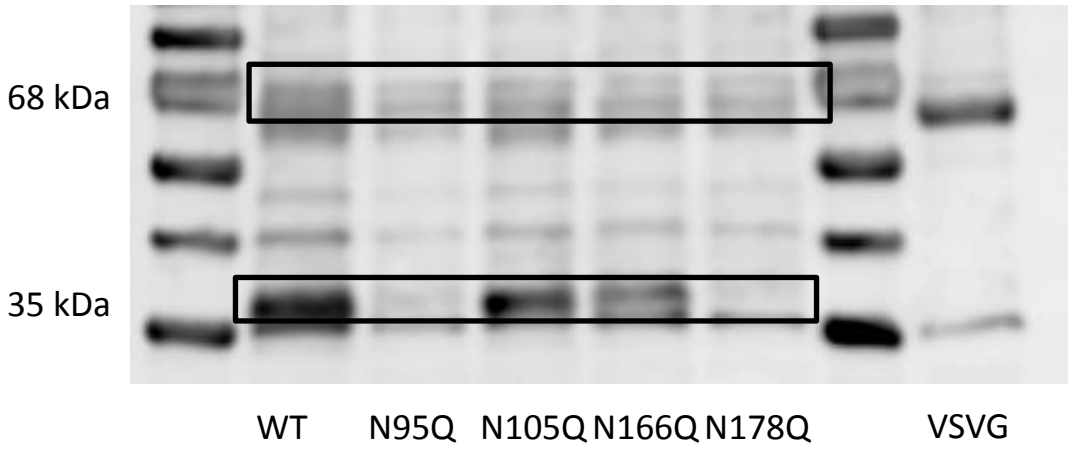

Fig. 4.5 Junín GPC is incorporated into MLV pseudovirus. WT and mutant GPC were transfected into HEK 293T cells along with the Gag-Pol packaging construct. Pseudovirion (JUNVpp) containing supernatants were harvest 48 h post-transduction and concentrated using PEG8000. The psv proteins were separated on a 10% SDS-PAGE gel and immunoblotted. GPC's were detected using immunostaining with polyclonal anti-myc. Cleaved GP-2 is outlined with a box at 35 kDa while uncleaved GPC is seen within the box at 68 kDa..

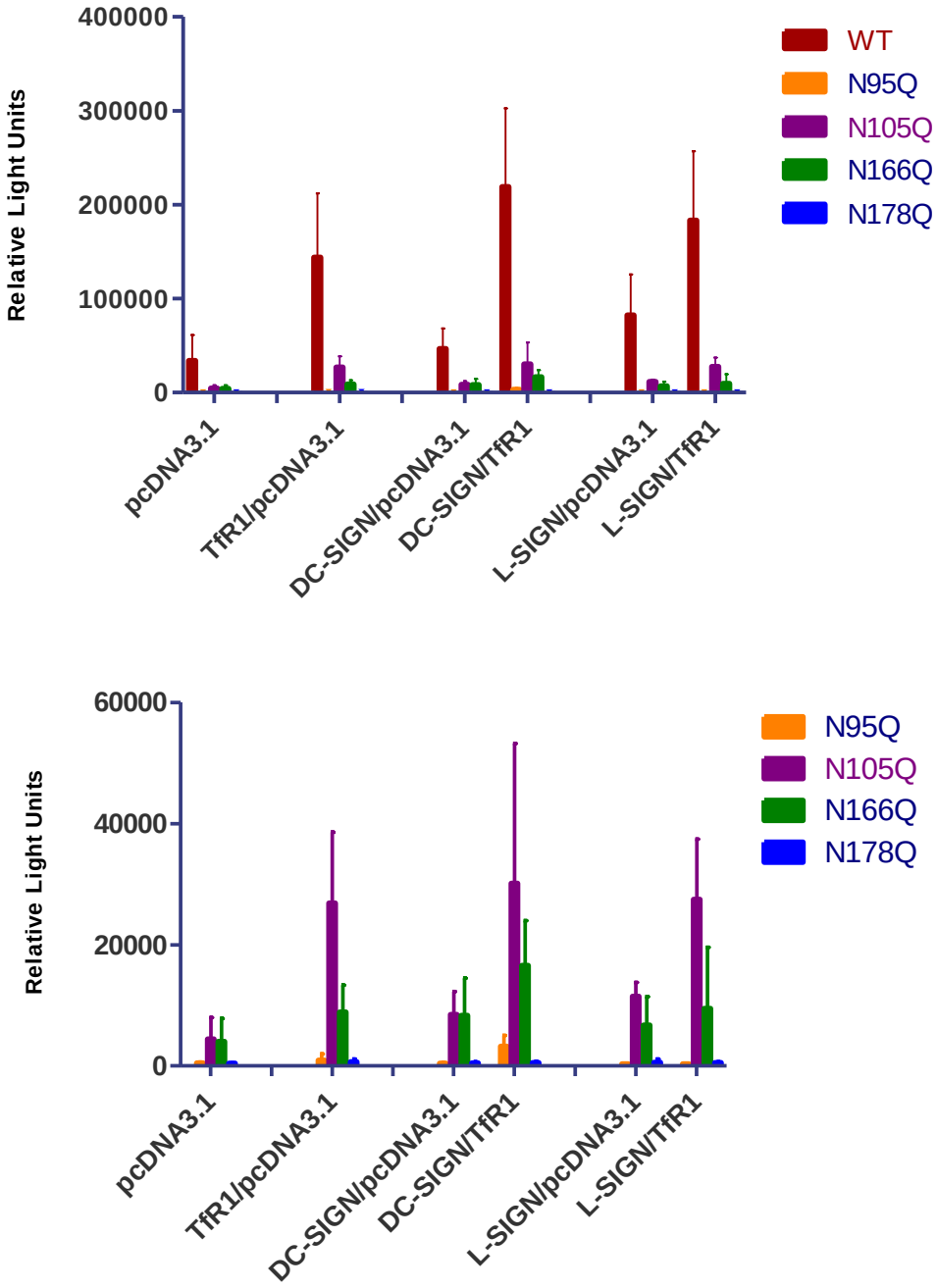

Fig. 4.6 Mutant JUNV-GPCpps have a limited ability to transduce. The human cellular receptors TfR1, L-SIGN and DC-SIGN were transfected into NIH-3T3 cells. The cells were transduced with WT or mutant JUNVpp. (A) WT and mutant GPCs transduction levels measured as a relative light unit. (B) The same data as (A) but with the WT removed to further analyze transduction levels. All data represent a minimum of 3 independent experiments.

Molecular modeling of GPCs.

WT and mutant GPCs were modeled onto the existing structure of Machupo Arenavirus GP-1 (9). The amended sequences containing the mutations and the WT GPC were sent to CPHmodels and visualized using Pymol (Fig. 4.7). There is 70% sequence homology between the aligned strains of Machupo and Junín Arenavirus glycoproteins and 54.8% homology between the GP-1s [data not shown] based on LAlign sequence analysis (28). Even though Junín WT and mutants were successfully modeled, I feel that based on the disparity in homology between the Machupo reference sequence and Junín that drawing conclusions based on the models would be speculative at best even though there is basic homology regarding the N-linked glycosylation sites. Based on the modeled Junín GP-1, residues N105 and N166 seem to be located on the surface of the structure

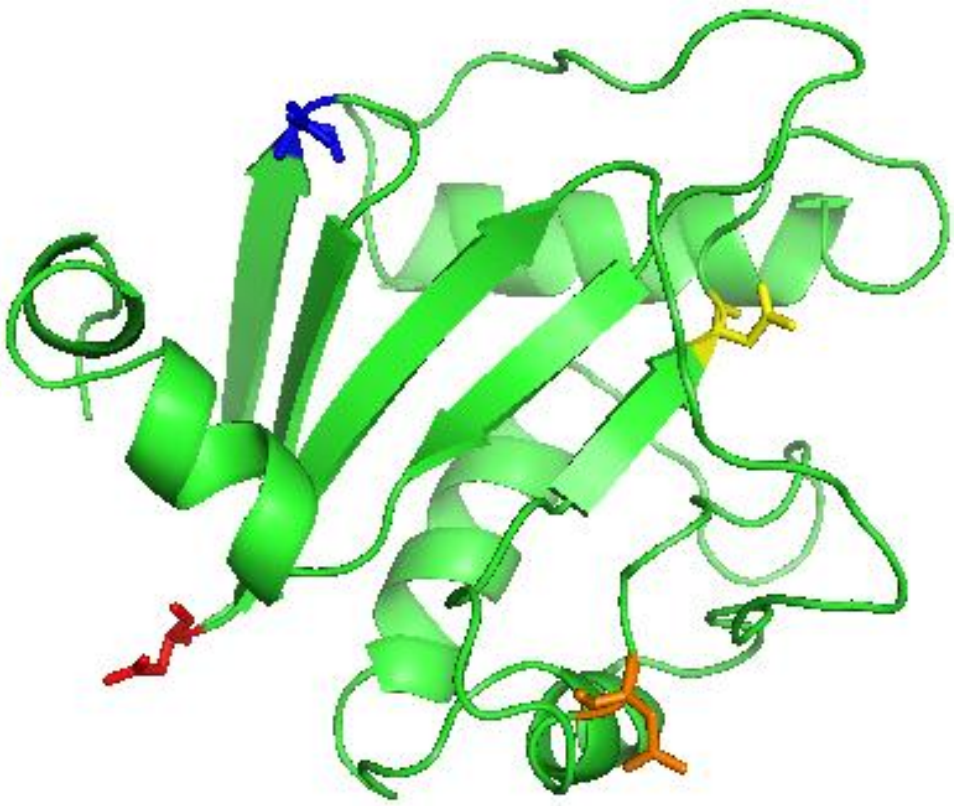

Fig. 4.7. Structure of Junín GP-1. Junín, strain IV4454, was submitted to CPHmodels using PDB 2WFO as a template. The resulting structure was modeled using Pymol. Asparagine residues are depicted as sticks. N95 is blue, N105 red, N166 orange, and N178 is yellow.

Discussion

Many viruses have glycosylated envelope glycoproteins including SARS, HIV, ebola, influenza and HCV which perform a number of roles involved in structure, entry and antigenicity (15, 25, 27, 29, 37). In this study, I analyzed the role of glycosylation of the GP-1 subunit of the Junín Arenavirus glycoprotein. GP-1 is involved in receptor binding and entry into host cells. Junín Arenavirus, strain IV4454, glycoprotein has 7 potential N-linked glycosylation sites: 4 sites on GP-1 and 3 on GP-2 (Fig. 4.1). Alignments between 5 different strains of Junín show remarkable homology between the predicted glycosylation sites with only the attenuated Candid1 and Romero strains lacking a site at N166 (Fig. 4.2). The lack of a glycan at N166 is not responsible for the attenuation of Candid1 (5).

Six of eight Old and New World arenaviruses have predicted glycosylation sites at the approximate positions of N166 and N178 based on NetNGlyc scoring parameters. I mutated the asparagines in the Asn-Xxx-Ser/Thr motif to glutamines effectively removing the ability of glycans to form. Removal of the glycan at these positions affects the ability of the glycoprotein to be cleaved (Fig. 4.4) and results in loss of infectivity (Fig. 4.6). A crystal structure for Machupo GP-1 has been solved and 4 potential glycosylation sites were identified (9). They report that all glycans are located on solvent accessible loops and N178 is located within a cavity. I believe that removal of the Asn at N178 affects the ability of this cavity to form which adversely affects the final shape.

Prediction of glycosylation does not mean that the site is occupied. N-linked glycans serve a role in protein expression, folding, transport and processing. Not all glycosylation sites are critical;

however, loss of particular crucial glycosylation sites may prevent the protein from obtaining the correct structure and ability to function (26, 35).

In this study, I demonstrated that all predicted GP-1 sites at N95, N105, N166, and N178 are glycosylated (Fig. 4.4). Removal of specific glycosylation sites appears to adversely affect structure as seen with the lack of GP-2 cleavage product for N95Q and N178Q and reduced cleavage product for position N166 (Fig. 4.4). The removal of N105 and N166 seems to affect function since there is reduced infectivity (Fig. 4.6) and to a lesser extent structure since cleaved GPC is incorporated into the pseudovirions (Fig. 4.5). Unfortunately, our assays do not identify if loss of function is due to improper folding.

Glycosylation sites on viral glycoproteins can bind to the carbohydrate recognition domains (CRDs) on cellular receptors. I chose 2 C-type lectins, DC-SIGN and L-SIGN, that bind to mannose since Junín GPC has mannose residues in an attempt to determine if the glycoprotein had any lectin affinity (22). Unfortunately, transduction levels were very low for all mutants when compared to WT JUNVpp ability to utilize either TfR1 or the lectins DC-SIGN and L-SIGN (Fig. 3.6A). Mutant N105 had the highest relative light units when compared to the other mutants (Fig. 3.6B). Mutants N95 and N178 had minimal transduction ability (Fig. 4.6A and B) which agrees with the inability of the proteins to be cleaved (Fig. 4.4) and included into pseudovirus particles (Fig. 4.5)

The results presented here suggest that all glycosylation sites on GP-1 are crucial for maintaining virus structure and ability to interact with potential receptors. This data is supported by the conservation we see of these residues across arenavirus strains. Since these residues are solvent

accessible, they may be involved in receptor binding. Additional assays that prevent the glycan from binding to a CRD on a receptor may help elucidate their role in virus entry.

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Chapter 5

Summary and Conclusion

The family *Arenaviridae* is divided geographically and phylogenetically into two major complexes, the New World and the Old World complex (2, 8). Seven of the known arenaviruses can cause viral hemorrhagic fever (VHF) in humans, with five of them, namely Junín, Guanarito, Machupo, Chapare, and Sabiá, belonging to the phylogenetic clade B of New World arenaviruses (3, 4, 7, 11). Rodents are the known reservoir of most arenaviruses and infection generally occurs after exposure to infected rodent excreta. Junín virus is the etiologic agent of Argentine Hemorrhagic Fever (AHF), an endemo-epidemic disease affecting populations living on the farming land of Argentina. To date, several thousand cases have been reported within the endemic regions with an average of 300+ new cases yearly (9, 19). AHF initially shows non-specific flu-like symptoms. As the disease progresses, hemorrhagic and neurological complications may occur with mortality rates ranging from 20-30% (18, 19).

Although a live attenuated vaccine, Candid1, is available against Junín, it is under-utilized and the endemic region is expanding, consequently the supplies of convalescent serum for treatment of infected people are decreasing. Furthermore there is a growing concern that these viruses could be used as agents of bioterrorism (12, 17). Therefore, the arenavirus family remains a serious threat to global public health.

Pathogenic New World arenaviruses can utilize the human transferrin receptor (TfR1) and to date that has been the only known receptor. In this thesis, I have identified three new receptors, LSECtin, DC-SIGN and L-SIGN that can be exploited by Junín to bind and enter susceptible cells.

In Chapter 2 I investigated Junín's interactions with the C-type lectins DC-SIGN and L-SIGN. C-type lectins have a carbohydrate recognition domain (CRD) that can bind select carbohydrates. C-type lectins can function as antigen receptors that can present antigens to other

cell types. DC-SIGN is an important receptor for dendritic cells and preferentially binds to high mannose structures found on viruses and bacteria (13, 14). However, viruses such as HIV, ebola and human cytomegalovirus have subverted dendritic cell machinery and instead *trans*-infect the cell rather than being destroyed and presented by MHC II (1, 6, 15). Data from Chapter 2 indicates that DC-SIGN and L-SIGN can function as attachment factor and alternative receptor respectively and both can enhance infection when expressed with TfR1.

I show that immature dendritic cells are trans-infected by JUNVpp. The pseudoparticle binds and associates with TfR1 and DC-SIGN on dendritic cells and is internalized to early endosomal compartments (EEA1) but is not seen progressing to the late endosomal/lysosomal compartment (LAMP-1). Progression to this low pH compartment is critical if the virus is to undergo fusion with the membrane thereby releasing the viral genetic material into the cytoplasm. Additional evidence that the virus does not progress to the point of membrane fusion is the lack of luciferase that is produced by the transduced cells. A luciferase reporter gene was included in the pseudoparticle as a measure of infectivity. Once the pseudoparticle undergoes fusion, luciferase will be produced and can be measured by a luminometer. Dendritic cells that were transduced with JUNVpp showed similar levels of luciferase activity as control un-transduced cells indicating that productive infection is not occurring.

Interestingly, dendritic cells can bind antigen to its neck region instead of the CRD. Binding in this region leads to antigen entering an alternative endocytic pathway where the antigen remains associated with early endosomal compartments and has delayed entry into lysosomal compartments. I believe that Junín Arenavirus is employing this mechanism and is effectively trans-infecting dendritic cells and using DC-SIGN as a recycling receptor instead of a more traditional endocytic and antigen processing receptor.

In Chapter 3, I resumed my study of alternative receptor use and continue my study of DC-SIGN and L-SIGN but also focused on LSECtin. DC-SIGN is found on dendritic cells and liver sinusoidal cells. L-SIGN and LSECtin are found on lymph nodes and liver sinusoidal cells. The lymph nodes and liver are primary sites of Junín Arenavirus infection. In the context of the liver, LSECtin acts as an antigen presenting cell and acts a barrier between blood borne pathogens and hepatocytes. Blocking DC-SIGN, L-SIGN and LSECtin receptors with antibodies significantly reduced infectivity in primary human liver sinusoidal cells (HHSEC) which express all three lectins and TfR1. Blocking LSECtin produced an effect that was similar to blocking TfR1 indicating that LSECtin is a true receptor of Junín infection. HHSEC cells are highly transducible with JUNVpp and as shown in Chapter 1 can be infected by trans-infected dendritic cells.

Building on data from Chapters 1 and 2 I propose a model of pathogenesis for Junín Arenavirus infection (Fig. 5.1). The established receptor, TfR1 is located basolaterally on lung epithelial tissue and is inaccessible to inhaled virus. I propose that sub-epithelial dendritic cells that sample the airway for antigens can bind and uptake virus but not be productively infected. This now trans-infected cell will enter a draining lymph duct and travel to a lymph node. The virus can then be transmitted to LSECtin, L-SIGN or TfR1 receptors in the lymph node and set up a productive infection. The virus can also travel to the other target organs such as the liver and spleen.

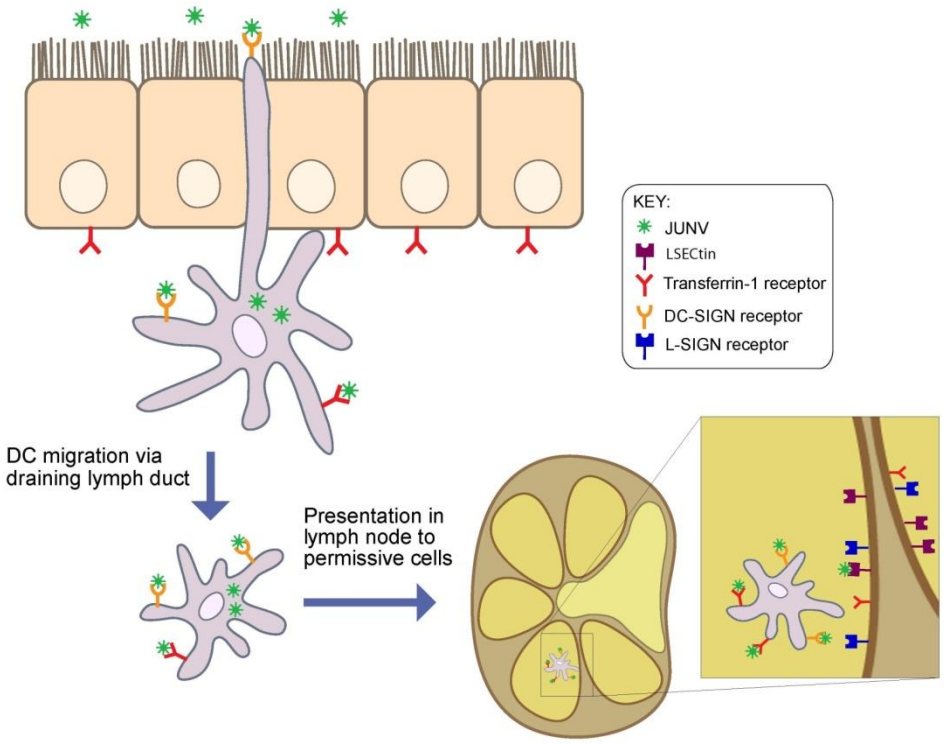

Fig. 5.1. Proposed Junín Pathogenesis Model. Junín Arenavirus uses the endogenous transferrin receptor of its human host to infect target cells and organs. It is believed that most infections arise from inhaling infected rodent excreta. However, the transferrin receptor is located basolaterally in the airway epithelium, which suggests that the virus may utilize additional receptor(s) to initiate infection. We propose that sub-epithelial dendritic cells, which are known to sample airway epithelium, bind JUNV via DC-SIGN, a C-type lectin used by many viruses, including HIV. Once JUNV binds DC-SIGN, the virus is internalized but does not set up a productive infection. Instead, the virus is transported to the lymph nodes and liver where the virus is then transmitted to receptors such as TfR1, LSECtin and L-SIGN.

In Chapter 4, I investigated N-linked glycosylation on the Junín glycoprotein complex (GPC) in an effort to determine if these sites are involved in binding and entry to a C-type lectin. GP-1 is involved in receptor binding and entry while GP-2 is involved in fusion with the endosomal membrane. Sequence analysis revealed that glycosylation is highly conserved on GP-2 across both Old and New World arenaviruses. GP-1 glycosylation is not as conserved but there are clusters across Families. I focused on Junín Arenavirus strain IV4454 which has 4 predicted sites on GP-1. The predicted sites of N95, N105, N166 and N178 were individually mutated to a glutamine to remove the glycosylation site. Mutants N95Q and N178Q failed to be cleaved but were still expressed on the cell surface; however, they were not included in pseudoparticles. Mutants N105Q and N166Q were cleaved and incorporated into JUNVpps. N105Q and N166Q inefficiently transduced NIH 3T3 cells transiently expressing TfR1, DC-SIGN and L-SIGN when compared to wild type JUNV GPC. Based on these data we believe that N95 and N178 are critical for glycoprotein structure while N105 and N166 are both involved in structure and entry. Glycosylation sites are important for proper folding of the protein and by removing sites we believe we changed the conformation of the protein. In the case of mutants N95Q and N166Q we believe we changed the conformation enough that the protein could not be cleaved in the Golgi.

Future Direction

Identification of dendritic cells being *trans*-infected by Junín Arenavirus is a large step in the field that needs to be investigated further. Some dendritic cells that are *trans*-infected with viruses transmit the viruses to T-cells which then become infected themselves (16, 20). Also, HIV work has shown that long term presence of the virus is required for eventual infection of dendritic cells which can then transmit the virus to T-cells (5, 10). To enhance our knowledge of Junín Arenavirus pathogenesis, experiments need to be conducted with T-cells to see if they can

be infected. It has been noted in human cases that there is a reduction in T-cells and that reduction could be explained by active infection of those cells.

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